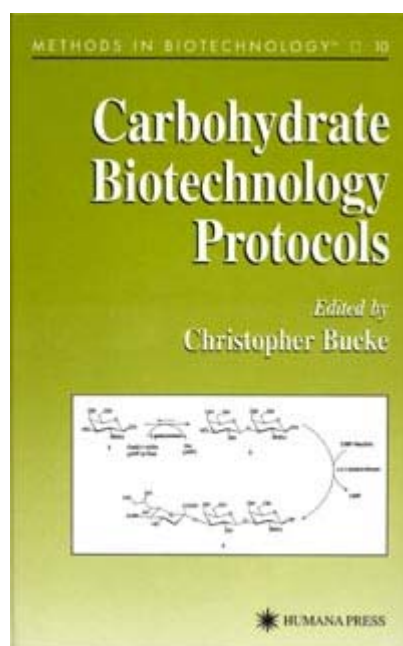


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METHODS IN BIOTECHNOLOGY™

Carbohydrate Biotechnology Protocols

Edited by
Christopher Bucke

*School of Biosciences,
University of Westminster, London, UK*

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Preface

We are in a phase of the evolution of biotechnology in which the true and potential commercial importance of carbohydrates is becoming appreciated more fully. Progress in providing hard facts to establish the commercial value of polysaccharides and oligosaccharides is limited, as always, by lack of funding and by a relative shortage of skilled practitioners in the production and analysis of those materials. Carbohydrate science has a reputation, not unmerited, for technical difficulty owing to the structural similarity of the many monosaccharide monomers and the potential, and real, complexity of oligosaccharides and polysaccharides, particularly heterosaccharides containing many different monomers. Modern analytical and synthetic methods, in many cases using enzyme technology, are beginning to allow this complexity to be unraveled.

Carbohydrate Biotechnology Protocols is aimed at those newcomers who have an interest in the production and use of carbohydrate materials, but have shied away from involvement for lack of detailed descriptions of appropriate methods, including the type of practical hints that may be provided by those skilled in those methods, but that are rarely described in research papers. The majority of the contributions to this book conform to the established format of the *Methods in Biotechnology* series. They begin with the theoretical and commercial background to the method or group of methods, provide a list of the reagents and equipment required for the procedure, then give a detailed step-by-step description of how to carry out the protocol. Each chapter concludes with a set of Notes, whose aim is to save the time of the user by indicating which problems are likely to arise and how best to deal with them.

The contributions begin with descriptions of methods for the production and isolation of microbial polysaccharides: the three polysaccharides selected—xanthan gum, microbial alginate, and schizophyllan—are of known commercial significance, and the methods described may be applied to the production of polysaccharides from other types or strains of microbes. The next three chapters describe the use of isolated enzymes in the synthesis and modification of polysaccharides (other than by hydrolysis). The core of the text concerns the production of smaller carbohydrate molecules, beginning

with the unusual cyclic oligosaccharides, cyclodextrins, and the microbial glycolipids, some of which have most unexpected structures with considerable commercial potential. There follow eight contributions on the production of oligosaccharides: this may seem excessive, but many different approaches are employed to produce a great diversity of materials, some inexpensive products for the food and animal feed industries, others very specialized structures for use by the pharmaceutical industry. Dr. Kren's contribution (Chap. 14) gives an example of the sophisticated chemo-enzymatic syntheses that are currently being developed for the preparation of complex oligosaccharides.

Enzymes coupled with physical methods, primarily electrophoresis, constitute the major means of determining the structures of nucleic acids. Until recently, analogous technology has not been available for the determination of carbohydrate structures. The FACE® method, described by Dr. Kumar and his colleagues (Chap. 18), remedies this lack.

The final group of chapters describes methods for producing and modifying low molecular weight carbohydrates. Dr. Elling's development (Chap. 19) of sucrose synthase-based syntheses of nucleotide sugars provokes optimism that the raw materials costs of producing oligosaccharides using "Leloir" glycosyltransferases may be lowered significantly. The later chapters describe, in general, the synthesis of simpler and cheaper products: sugars, sugar alcohols, and sugar derivatives. Finally, there is description of the use of an enzyme in the development of flavors in wines and fruit juices, a subject that will be of interest to those concerned with attempting to accelerate the maturation of wines.

It is intended that *Carbohydrate Biotechnology Protocols* should be readable and readily intelligible. As a consequence, it should be of interest and value to synthetic chemists, fermentation technologists, and applied enzymologists seeking information on the application of techniques—some familiar, some novel and very advanced—to produce compounds that may seem out of the ordinary. Most of all, it is hoped that this book will contribute to the advance of carbohydrate science, which is perhaps the last great area of molecular biology to be explored.

CHRISTOPHER BUCKE

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Contributors

Alain Arnaud • *Chaire de Microbiologie Industrielle et de Génétique des Micro-organismes, Ecole Nationale Supérieure Agronomique de Montpellier, Montpellier, France*

Begoña Bartolome • *Biochemistry Department, Institute of Food Research, Norwich Research Park, Norwich, UK*

Emmanuel Boures • *ULICE, Zac "Les Portes de Riom," Riom, France*

Marie-Pierre Bousquet • *Centre de Bioingénierie Gilbert Durand, Département de Génie Biochimique et Alimentaire, INSA, UMR CNRS, LA INRA, Complexe Scientifique de Rangueil, Toulouse, France*

Klaus Buchholz • *Department of Carbohydrate Technology, Technical University of Braunschweig, Braunschweig, Germany*

Christopher Bucke • *School of Biosciences, University of Westminster, London, UK*

José A. Casas • *Departamento de Ingeniera Química, Facultad Ciencias Químicas, Universidad Complutense, Madrid, Spain*

Peter S. J. Cheetham • *Zylepsis Ltd, Ashford, Kent, UK*

Patrick Chemardin • *Département de Microbiologie Industrielle et de Génétique des Micro-organismes, Ecole Nationale Supérieure Agronomique de Montpellier, Montpellier, France*

Stephen P. Church • *Ilex Close, Sonning Common, Reading, UK*

Francesca Clementi • *Dipartimento di Biotecnologie Agrarie ed Ambientali, Università degli Studi di Ancona, Ancona, Italy*

Patricia A. Denny • *Biotechnology Group, Hyland Division, Baxter Healthcare Corporation, Duarte, CA*

Paul C. Denny • *Biotechnology Group, Hyland Division, Baxter Healthcare Corporation, Duarte, CA*

Marguerite Dols • *Centre de Bioingénierie Gilbert Durand, Département de Génie Biochimique et Alimentaire, INSA, UMR CNRS, LA INRA, Complexe Scientifique de Rangueil, Toulouse, France*

Michael J. Donnelly • *AKZO NOBEL UK, plc, Coventry, UK*

Lothar Elling • *Institute of Enzyme Technology, Heinrich-Heine-University Düsseldorf, Research Center Jülich, Jülich, Germany*

Helga Erstesvåg • *The Norwegian Polymer Laboratory, Institute of Biotechnology, Norwegian University for Science and Technology, Trondheim, Norway*

Craig B. Faulds • *Biochemistry Department, Institute of Food Research, Norwich Research Park, Norwich, UK*

Pierre Galzy • *Département de Microbiologie Industrielle et de Génétique des Micro-organismes, Ecole Nationale Supérieure Agronomique de Montpellier, Montpellier, France*

Félix García-Ochoa • *Departamento de Ingeniera Química, Facultad Ciencias Químicas, Universidad Complutense, Madrid, Spain*

Yannick Gueguen • *IFREMER, Centre de BREST, Laboratoire de Biotechnologie, Plouzané, France*

Guilhem Janbon • *Département de Microbiologie Industrielle et de Génétique des Micro-organismes, Ecole Nationale Supérieure Agronomique de Montpellier, Montpellier, France*

Jeffrey A. Khan • *Department of Macromolecular Sciences, Institute of Food Research, Earley Gate, Reading, UK*

Dong Hyun Kim • *Department of Biotechnology, Taegu University, Kyungbuk, Korea*

Shiro Kobayashi • *Department of Materials Chemistry, Graduate School of Engineering, Kyoto University, Kyoto, Japan*

Vladimir Kren * • *Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídenská, Prague, Czech Republic*

Paul A. Kroon • *Biochemistry Department, Institute of Food Research, Norwich Research Park, Norwich, UK*

Harish P. M. Kumar • *Biotechnology Group, Hyland Division, Baxter Healthcare Corporation, Duarte, CA*

Siegmund Lang • *Department of Biochemistry and Biotechnology, Technical University Braunschweig, Braunschweig, Germany*

Leon M. Marchal • *Department of Food Technology and Nutrition Science, Food and Bioprocess Engineering Group, Wageningen Agricultural University, Wageningen, The Netherlands*

Anna Millqvist-Fureby • *Department of Macromolecular Sciences, Institute of Food Research, Earley Gate, Reading, UK*

Vincent Monchois • *Centre de Bioingénierie Gilbert Durand, Département de Génie Biochimique et Alimentaire, INSA, UMR CNRS, LA INRA, Complexe Scientifique de Rangueil, Toulouse, France*

Pierre F. Monsan • *Centre de Bioingénierie Gilbert Durand, Département de Génie Biochimique et Alimentaire, INSA, UMR CNRS, LA INRA, Complexe Scientifique de Rangueil, Toulouse, France*

Mauro Moresi • *Istituto di Tecnologia Agroalimentari, University of Tuscia-Viterbo, Viterbo, Italy*

Riccardo A. A. Muzzarelli • *Faculty of Medicine, Università degli Studi di Ancona, Ancona, Italy*

John Packwood • *School of Biosciences, University of Westminster, London, UK*

Eugenio Parente • *Dipartimento di Biologia Difesa e Biotecnologie Agro Forestali, Università degli Studi Della Basilicata, Potenza, Italy*

François Paul • *Centre de Bioingénierie Gilbert Durand, Département de Génie Biochimique et Alimentaire, INSA, UMR CNRS, LA INRA, Complexe Scientifique de Rangueil, Toulouse, France*

Viviana Ramos • *Faculty of Medicine, Università degli Studi di Ancona, Ancona, Italy*

Robert A. Rastall • *Department of Food Science and Technology, University of Reading, Whiteknights, Reading, UK*

Udo Rau • *Biotechnology Group, Department of Biochemistry and Biotechnology, Technical University Braunschweig, Braunschweig, Germany*

Magali Remaud-Siméon • *Centre de Bioingénierie Gilbert Durand, Département de Génie Biochimique et Alimentaire, INSA, UMR CNRS, LA INRA, Complexe Scientifique de Rangueil, Toulouse, France*

Jacob A. Rendleman, Jr. • *Biopolymer Research Unit, National Center for Agricultural Utilization Research, USDA Agricultural Research Service, Peoria, IL*

Victoria E. Santos • *Departamento de Ingeniera Química, Facultad Ciencias Químicas, Universidad Complutense, Madrid, Spain*

Shin-Ichiro Shoda • *Department of Materials Chemistry, Graduate School of Engineering, Tohoku University, Aoba, Sendai, Japan*

Gudmund Skjåk-Bræk • *The Norwegian Polymer Laboratory, Institute of Biotechnology, Norwegian University for Science and Technology, Trondheim, Norway*

Wim Soetaert • *Laboratory of Industrial Microbiology and Biocatalysis, University of Ghent, Ghent, Belgium*

Seung Koo Song • *Department of Chemical Engineering, Pusan National University, Pusan, Korea*

Vesna Stanic • *Faculty of Medicine, Università degli Studi di Ancona, Ancona, Italy*

Eberhard Stoppok • *Zuckerinstitut, Braunschweig, Germany*

Sony Suwasono • *Department of Food Science and Technology, University of Reading, Whiteknights, Reading, UK*

Yukio Suzuki • *Division of Biochemistry, Research Institute for Bioresources, Okayama University, Kurashiki, Japan*

Kei Uchida • *Division of Biochemistry, Research Institute for Bioresources, Okayama University, Kurashiki, Japan*

Erick J. Vandamme • *Laboratory of Industrial Microbiology and Biocatalysis, University of Ghent, Ghent, Belgium*

Petra T. Vanhooren • *Laboratory of Industrial Microbiology and Biocatalysis, University of Ghent, Ghent, Belgium*

Evgeny N. Vulfson • *Department of Macromolecular Sciences, Institute of Food Research, Earley Gate, Reading, UK*

René-Marc Willemot • *Centre de Bioingénierie Gilbert Durand, Département de Génie Biochimique et Alimentaire, INSA, UMR CNRS, LA INRA, Complexe Scientifique de Rangueil, Toulouse, France*

Gary Williamson • *Institute of Food Research, Norwich Laboratory, Norwich Research Park, Norwich, UK*

Jong Won Yun • *Department of Biotechnology, Taegu University, Kyungbuk, Korea*

Astrid Zervosen • *Institute of Enzyme Technology, Heinrich-Heine-University Düsseldorf, Research Center Jülich, Jülich, Germany*

1—

Introduction to Carbohydrate Biotechnology

Christopher Bucke

The study of carbohydrates has been neglected in favor of consideration of investigation of the properties and functions of the more obviously important classes of biochemicals, nucleic acids, and proteins. There are various reasons for this: the concept of carbohydrates as relatively boring materials that are important as major food components and as structural molecules in cell walls but without subtlety of function, as well as the sheer difficulty of working with them. During the evolution of molecules the new availability of monosaccharides provided nature with a class of molecules that could be used as the building blocks for macromolecules of a complexity far in excess of that which can be achieved by polymerizing amino acids. With four (in pentoses) or five (in hexoses) hydroxyl groups available for reaction on each monomer, polymers with various degrees of branching are possible using only a single sugar as monomer, but some 25 different monomers actually occur in naturally occurring carbohydrates, making a staggering, unimaginable number of combinations possible. Carbohydrates thus can act as the repositories of enormous amounts of information: elucidation of the language in which this information is coded may well occupy biotechnologists and experts in linguistics well into the next century. They will be thankful that not all the sugar structures possible are actually used in biological molecules, but they will have to remain aware that nature has tools available that have not been employed (probably!).

This information lies on the surface of biological molecules, in the complex oligosaccharides *N*-linked or *O*-linked to proteins: It should not be forgotten that the majority of proteins of eukaryotic organisms are glycosylated. As the oligosaccharides are hydrophilic, they occur usually on surfaces, that is, those parts of molecules that first come into contact with other molecules. As they are hydrophilic they are usually associated with large amounts of water and it

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may be that those aiming to elucidate the language(s) borne on carbohydrates will have to evaluate the ways in which water structure is affected by carbohydrates.

This is speculation about methods to be used in the future: this volume concerns methods in current use, some of which are of considerable complexity. In selecting chapters for such a volume as this, an editor has several problems to resolve. First is the definition of the subject, what to include and what to exclude. Biotechnology has been described as “the application of biological and engineering principles to the provision of products and services” (**1**), so this volume concerns itself with methods by which carbohydrate products may be made and to a lesser extent with the provision of services. Second, the editor has to decide whether some processes are so well-established that there would be little point in providing another account of material that is readily available elsewhere. The reader will not find details of how to use cellulases to hydrolyze cellulose or of how to use invertase to hydrolyze sucrose because that information is readily available elsewhere (e.g., **ref. 2**). The temptation to include an account of the use of glucose isomerase to convert glucose into high fructose syrup has been resisted simply because it seems improbable that there would be any need to conduct this commercially important process on the laboratory scale and because adequate descriptions of the process are available in other volumes (e.g., **ref. 3**). Enzymes play a crucially important part in this volume but it is the application of enzyme technology that is described, not the preparation of carbohydrate modifying enzymes, which is a potential topic for another volume. So this volume concerns itself with the products of processes using fermentation and enzyme technology. It does not claim to provide a comprehensive account of all the modern processes in use and under development but an aim is to provide representative examples of at least one of the different types of process that are used. In compiling such a volume the contents are dictated to some extent by the willingness of the key experts to devote valuable time to the preparation of chapters. The editor's sincere thanks are offered to the various contributing authors, and especially to those who volunteered to produce chapters in addition to those originally requested. This has resulted in the book being significantly more up to date than would have otherwise been the case.

Fermentation Processes

Anyone who has streaked a soil extract onto agar plates containing a medium rich in carbohydrate will realize that a very large proportion of the bacteria in the extract are capable of producing polysaccharides, some in very large quantities. Screening the colonies to determine the potential commercial value of the polysaccharides is less easy and generally frustrating because the majority of them provide only viscosity. A relatively few microbial polysaccharides are

produced in significant quantities and methods for the production and quality assessment of some of those are presented in Chapters 2 to 4. Applications of microbial polysaccharides have been reviewed recently (4). Rather fewer microbes have the ability to produce surface-active agents but many of the microbial surfactants that are produced contain carbohydrates, often in complex and improbable structures, as Dr Lang's Chapter 9 describes.

Enzymatic Processes

Plant derived polysaccharides are, in general, more familiar than microbial polysaccharides but value can be added to them by structural modification, chemical, as in the preparation of the propylene glycol alginate used to maintain the "head" on certain types of beer (in the United Kingdom), or enzymatic. The simplest polysaccharide modifications are hydrolyses, which often produce valuable products, but other improvements may be achieved by specific oxidation, as Dr. Donnelly describes (Chap. 7). A particularly exciting development is the use, detailed by Professor Skjak-Braek (Chap. 6), of polymannuronate epimerase, cloned from *Azotobacter vinelandii* into *Escherichia coli* to increase the content of guluronate in alginate derived from brown algae. This allows the alginate to be used to entrap islets of Langerhans for implantation into diabetic animals (and, potentially, humans), polymannuronate but not polyguluronate having antigenic properties.

The antigenic properties of polymannuronate are not unusual and there is a rapidly growing body of information describing the use of oligosaccharides and polysaccharides to elicit or enhance the development of defense mechanisms in animals, plants, and microbes. Progress in the exploration of this topic is hampered by the cost, rarity, or nonavailability of characterized oligosaccharides. Chemical synthesis of all but the simplest oligosaccharides is so complex as to be prohibitively expensive, as is the isolation of oligosaccharides from natural sources. Enzymatic synthesis is much more practical and feasible, if not generally inexpensive, and several authors (Chaps. 10–17) provide methods for oligosaccharide synthesis starting from readily accessible materials such as polysaccharides and monosaccharides. The less expensive oligosaccharides, such as some glucans and all fructans derived from sucrose or inulin, find uses as "functional foods," passing unchanged into the large intestine where they favor the growth of beneficial microbes, principally Bifidobacteria.

Enzymes whose role is to catalyze the hydrolysis of carbohydrates may be considered as transferases whose normal second, acceptor, substrate is water. Many of them can use a wide variety of materials as alternative acceptors so they can catalyze the synthesis of oligosaccharides, alkyl glycosides, and glycosides of other materials with hydroxyl groups. Considerable ingenuity has

been demonstrated by enzyme technologists in developing means of increasing the yields of such syntheses. Various oligosaccharides are potential candidates as carbohydrate drugs (5), and enzymatic synthesis is the most appropriate means of producing these. A key stage in the synthesis of oligosaccharides for commercial use is the provision of inexpensive starting materials. Dr. Elling (Chap. 19) describes the use of sucrose synthase as an initial stage to produce nucleotide sugars relatively inexpensively.

Huge numbers of carbohydrate-modifying enzymes are known, often differing very subtly in their specificities: a fascinating topic for future molecular biologists is to determine the molecular bases for these slight variations. Suitably characterized enzymes are of great value in determining the structures of oligosaccharides and polysaccharides: Dr. Kumar and his colleagues (Chap. 18) describe such applications.

Novel Sugars and Sugar Derivatives

The food industry is always keen to explore the functionality of new materials, provided that they are impeccably safe and inexpensive. Microbes have various enzyme systems that allow the synthesis of novel, or underexploited, materials, two of which are described in Chapters 20 and 21. Once novel materials are available in quantity, “second generation” processes may be developed: In Chapter 22, Dr. Stoppok and Professor Buchholz describe the enzymatic modification of disaccharides to enhance their potential use as starting materials for further synthesis.

The normal role of some glycosidases is apparently not to hydrolyze carbohydrates but to remove sugar residues from the glycosides of nonsugar aglycones, of which plant tissues contain a multiplicity. Recent studies have shown that glycosides of volatile aglycones frequently occur in fruit juices, so controlled hydrolysis to release the aglycones may speed some aspects of the maturation of wines, thus providing a potentially significant service to the wine industry. Dr. Gueguen and his colleagues (Chap. 26) describe how this may be achieved. In the appropriate conditions, glycosidases may be used “in reverse” to synthesize glycosides that are novel or difficult to produce chemically. Chapters 23–25 give examples of the application of novel enzyme technologies to the synthesis of glycosides.

Carbohydrate biotechnology is still in its infancy. An aim of this volume is to provide information that may speed its attainment of greater maturity, without passage through too troubled an adolescence.

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2—

Production and Isolation of Xanthan Gum

Félix García-Ochoa, Victoria E. Santos, and José A. Casas

1—

Introduction

Manufacture of high-molecular-weight compounds with thickener properties has been traditionally related to plants, seeds, and seaweeds. These compounds have been named gums. The rheological properties of their solutions show important alterations depending on uncontrolled variables such as weather, and their manual-collection labor cost can often influence their market price.

Production of molecules with thickener properties from microorganisms was an important advance. This production is made under control and the polymer has constant properties. Xanthan gum is one of these biopolymers first commercialized in the 1960s, and since then has played an important role in industrial gum applications **(1)**.

Xanthan gum is a polysaccharide synthesized by *Xanthomonas* sp. Its structure can be seen in **Fig. 1**. The repetition of this structural unit forms xanthan molecules showing very high molecular weights of several millions of Daltons. The acetyl and pyruvyl contents can change depending on culture conditions and microorganism used **(2)**. Therefore, the polymer solutions show different rheological behavior, depending on molecular weight and composition. Xanthan with a high pyruvate content (4–4.8%) shows a greater thickener behavior than that with low pyruvate content (2.5–3%) **(1)**. Pyruvate-free xanthan is employed in enhanced oil recovery (EOR) because microgels are not formed, although in other applications this is not so important. Xanthan's solubility in water and its high stability and thickener behavior, together with the simplicity of its industrial manufacture, made this polysaccharide a gum frequently employed for water rheological behavior modification in many industries such as food, pharmaceutical, and cosmetic and also in EOR.

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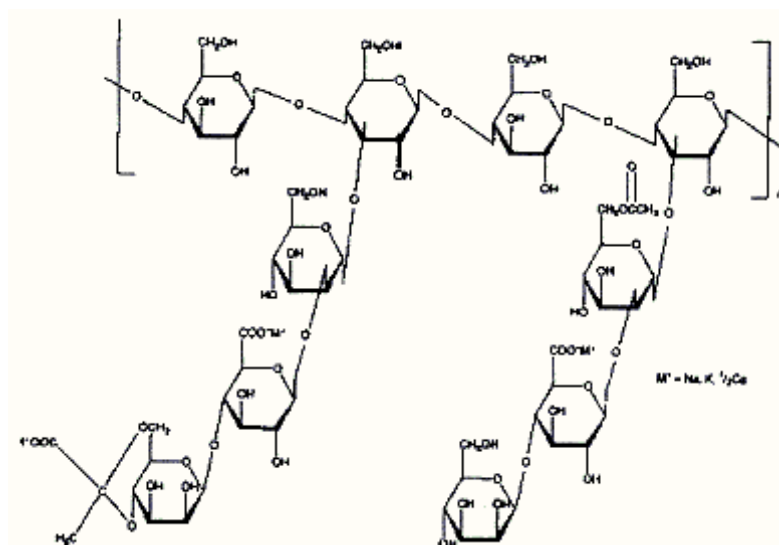


Fig. 1.
Xanthan molecule structure.

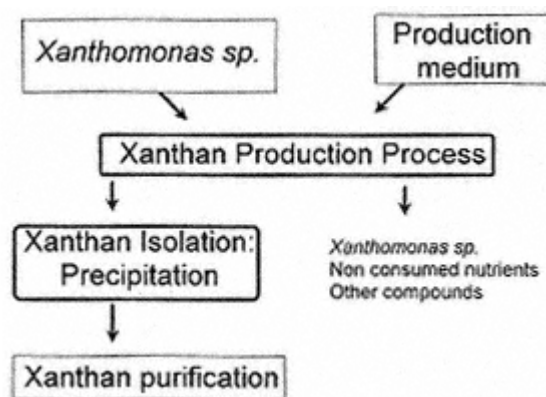


Fig. 2.
Xanthan gum process scheme.

A xanthan production scheme is given in **Fig. 2**. Two different steps can be considered: production and isolation. In this chapter both will be commented on separately.

1.1—

Xanthan Production

The type of xanthan gum produced is quite different depending on the *Xanthomonas* species used, the production medium composition, and the

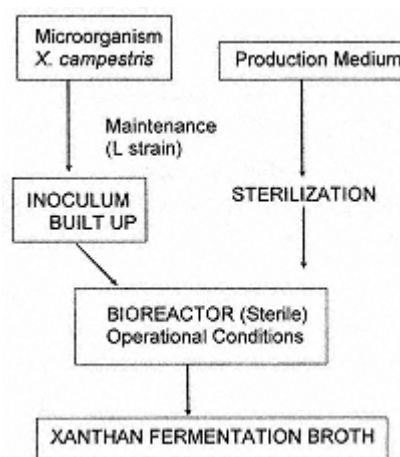


Fig. 3.
Xanthan production scheme.

operational conditions employed in the fermentation (2). Most bacteria of the *Xanthomonas* genus produce extracellular polysaccharides as bacterial capsules (3). The different composition of these gums is related to their contents of glucose, glucuronic acid, mannose, pyruvate, and acetate, and another sugar, galactose, is introduced into the molecule by some species (2). The type of xanthan gum produced is also influenced by the operational conditions (such as temperature, pH, dissolved oxygen, and so on) employed during the process, both in the concentration or yield obtained and in its molecular structure. The media composition seems to influence the pyruvate content, and operational conditions employed in the fermentation (mainly temperature and dissolved oxygen concentration) influence the molecular weight of the product obtained (4–8).

Xanthan gum production needs several previous steps to be carried out successfully (Fig. 3). The microorganism has to be maintained in a viable form (strain maintenance) to be grown in a complex medium to build up an inoculum able to produce the gum by fermentation. All of these steps are described in depth in this chapter.

1.2—

Isolation of Xanthan Gum

A typical xanthan production fermentation broth after 60–90 h, depending on the strain, medium composition, and operational conditions is composed of 1–3% (w/w) xanthan gum, 0.1–0.3% (w/w dried) *Xanthomonas* cells, 0.1–1 % (w/w) unused carbohydrate, salts, and other medium components. Thus, in xanthan downstream processing it is necessary to eliminate up to 95% of the fermentation broth. The objectives of downstream processing are:

- Extraction of polymer in a solid form, that is, stable and easy to handle, store, and dissolve.
- Separation of insoluble solids precipitated together with xanthan.
- Deactivation of enzymes able to degrade xanthan molecules.

According to these objectives there are different processes for the isolation of xanthan gum. These usually involve both physical and chemical separation steps **(2)**, such as:

- Preliminary treatments for degradation and removal of the cells.
- Polysaccharide precipitation.
- Final steps, including washing, dewatering, drying, milling, and packing.

Preliminary treatments used are of thermal or chemical nature. Usually after the xanthan production process cells are lysed by a temperature increase, which also favors xanthan dissolution and decreases broth viscosity. Cells are then separated from the production broth by filtration or centrifugation. Another possibility is the use of water or chemical agents such as alcohols to dilute the xanthan fermentation broth, decreasing viscosity and allowing cell removal by filtration. When chemical reagents such as alcohols or ketones are used, cells are also lysed and xanthan separation is enhanced.

After cell removal, xanthan in solution can be isolated by precipitation, decreasing its solubility by the addition of organic chemical agents or polyvalent salts, producing xanthan polyelectrolyte neutralization and precipitation. Combinations of both agents can be used. Precipitation is a simple technique commonly used for recovering many biological products such as antibiotics, proteins and biopolymers. The tendency of these substances to precipitate is governed by many factors: solvent environment (e.g., salt concentration, dielectric constant, and pH), temperature, and the size, shape, and charge of the molecules. One of the most common strategies to induce precipitation is to alter solvent properties, for example, by adding a miscible organic solvent or an electrolyte **(9)**. Low-molecular-weight alcohols or ketones such as methanol, ethanol, isopropanol, *t*-butanol, and acetone are the organic solvents more usually employed in xanthan precipitation **(10)**. Isopropanol (IPA) is used most frequently **(1,10,11)**, with a ratio between 1.8:1 to 2.5:1 (v/v, IPA/broth) being employed. Normally an excess of 8–25% of this quantity is used for washing. Recovery of the alcohol is essential for process economic viability **(1)**.

Other authors **(10–12)** have proposed to use polyvalent cations for polysaccharide precipitation. Xanthan can be precipitated by addition of calcium salts **(10)** under basic pH (between 8.5 and 12), or by aluminum salts addition **(12)**. In this way, an insoluble xanthan salt is obtained that must be converted to a soluble salt (sodium or potassium) for commercial uses **(12)**. Other authors have proposed to employ quaternary ammonium salts **(11,12)**,

but finally the product must also be converted to a sodium or potassium salt, and the xanthan obtained in this way is not eligible for food industry uses (2) due to the toxicity of quaternary ammonium salts and also because of their high cost.

Smith (13) and García-Ochoa et al. (10) have described the joint utilization of alcohol and salt for xanthan precipitation, finding that smaller quantities of the agents are needed. A general description of the xanthan precipitation procedure is given below.

Xanthan gum that is finally obtained must fulfill commercial quality parameters, such as: acetate and pyruvate contents, ash and moisture contents, and viscosity of its aqueous solutions, the main characteristic for thickener applications.

2—

Materials

2.1—

Xanthan Production

The main material employed in xanthan gum production is the bacteria itself: *Xanthomonas campestris* (NRRL B-1459). This microorganism can be obtained as a lyophilized sample from the following: Microbiology Culture Collection Research, Fermentation Laboratory of U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604.

1. YM medium: 10 g/L D-glucose purissimo; 5 g/L bacteriological peptone; 3 g/L yeast extract; 3 g/L malt extract. When YM-agar medium is employed, Agaragar purissimo (20 g/L) must be added. Bacteriological peptone, yeast extract and malt extract have to be stored at 4°C.

2. YM-T medium: 12 g/L D-glucose purissimo, 2.5 g/L bacteriological peptone, 1.5 g/L yeast extract, 1.5 g/L malt extract, 1.5 g/L $\text{PO}_4\text{H}(\text{NH}_4)_2$, 2.5 g/L PO_4HK_2 , and 0.05 g/L MgSO_4 . The pH has to be adjusted to 7.0 by addition of HCl.

3. Production medium (8): 40 g/L sucrose, 2.1 g/L citric acid, 1.144 g/L NH_4NO_3 , 2.866 g/L KH_2PO_4 , 0.507 g/L MgCl_2 , 0.089 g/L Na_2SO_4 , 0.006 g/L H_3BO_3 , 0.006 g/L ZnO, 0.020 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.020 g/L CaCO_3 , and 0.13 mL/L HCl cc. The pH has to be adjusted to 7.0 by adding NaOH. All the products must be pure.

2.2—

Materials Used in Xanthan Gum Isolation

Fermentation broths obtained as previously described were precipitated using several agents (10) such as: ethanol, IPA, and acetone, all of industrial quality, that is 96% (w/w), 85% (w/w), and 98% (w/w), respectively, and salts (NaCl, CaCl_2) of pure quality, around 99% (w/w) in purity.

Characterization of xanthan produced was performed by the measurement of different parameters. Acetate and pyruvate content were measured using enzymatic kits, acetate using Boehringer-Mannheim no. 148261 and pyruvate with Boehringer-Mannheim no. 124982. Rheological behavior was determined using a viscosimeter (Brookfield LVT-Synchroelectric). This viscosimeter has

a microcapsule for sample thermostation (Brookfield SC4-18/13R). A Brookfield no. 18 spindle was usually employed. Ash and moisture contents in xanthan were measured using a Dupont 951 thermogravimetric analyzer.

3—

Methods

3.1—

Procedure for Xanthan Production.

Several steps must be followed to carry out xanthan gum production successfully: strain maintenance, inoculum buildup, and xanthan production itself. The evolution of the production system can be followed according to the different analysis techniques that are described at the end of this section.

3.1.1—

Strain Maintenance

The first step in xanthan gum production is to ensure that the strain to be used in fermentation is L-strain (the real gum producer). The microorganism has to be plated on YM-agar plates and incubated at 25°C for 3 d. The colonies obtained must be bright yellow and between 4 and 5 mm diameter. To avoid degradation of L-strain to strains Sm (2–3 mm diameter) and Vs (1 mm diameter), not bright and pale yellow, *X. campestris* has to be transferred to fresh YM-agar medium every 14 d and incubated under the same conditions described above (4,14).

3.1.2—

Inoculum Buildup

The size of the inoculum to be employed is around 5–10% of the total fermentation volume. The microorganism state to be used for production has a great influence on overall process evolution rate. In xanthan fermentation this step is really important, because xanthan is the bacterial capsule of *X. campestris*, and the gum is produced when the microorganism is growing. The xanthan gum produced during this stage is not welcome because of its great resistance to nutrient uptake by the cells. The culture needed for inoculation for production has to be in the exponential growing phase and must have an important biomass concentration without xanthan gum. This inoculum must be built up in different stages, that is, from different growth cycles. When the final fermentation volume is around 2 L, two stages are enough. (1) The protocol for inoculum buildup (for a final vol of 2 L) can be carried out in an orbital shaker as follows: four 15-mL tubes with 7 mL of YM sterile media are inoculated with a loop of the bacteria less than 3 d old and incubated in a shaker at 28°C for 12 h. (2) The contents of each tube are introduced into a 250-mL Erlenmeyer flask containing 43 mL of YM-T sterile medium (see **Note 1**) (this process has to be carried out in a vertical laminar-flow work station) and incubated at 28°C for 6 h (7). (3) The stages for inoculum buildup have to be

increased as the final fermentation volume is higher in order to avoid xanthan gum production during growth. These stages are also useful to allow the bacteria to adopt to the new culture conditions such as different medium composition, mechanical stirring, and so on.

3.1.3— Xanthan Production

The production stage can be divided into three steps: fermentor sterilization and preparation, inoculation, and fermentation itself.

3.1.3.1— *Fermentor Sterilization and Preparation*

1. The concentrated medium without carbon source is sterilized in the vessel and the concentrated solution of the carbon source (sucrose) is sterilized separately and afterwards introduced into the vessel (see **Note 2**).
2. The pH electrode must be calibrated before fermentor sterilization.
3. All the exits, inputs and electrodes of the fermentor have to be sealed to avoid any problems with water stream during sterilization.
4. The sterilization has to be carried out at 121°C for at least 20 min.
5. After the sterilization, the control of temperature is turned on and the sugar (sucrose) is introduced into the vessel (see **Note 2**).
6. When the temperature value is close to the set point (28°C), the oxygen electrode is switched on, being previously polarized for at least 6 h. Afterwards it has to be calibrated by means of oxygen desorption with nitrogen and absorption with air employing the stirrer speed of the first stage in the fermentation (for 2 L of final work vol the initial stirrer speed is around 210 rpm).
7. The operational conditions described have been optimized to obtain high xanthan concentrations (**7**).

3.1.3.2— *Inoculation*

1. The microorganism is introduced into the vessel when all the operational conditions have been established. The volume of the inoculum that is to be introduced at the beginning of the fermentation to obtain a predetermined biomass concentration can be calculated from the value of the inoculum concentration. To determine the biomass content in the inoculum, the cultures in the different Erlenmeyer flasks have to be mixed in a vertical laminar work station in a sterile way. A small sample of the mixed culture is analyzed by means of a spectrophotometer at 540 nm to know the biomass concentration (see **Subheading 3.1.4.1.**). The volume of inoculum to be employed for fermentation is calculated from the concentration obtained.
2. The culture is introduced throughout a membrane employing a sterile syringe.

3.1.3.3— *Fermentation*

1. The fermentation takes place when inoculum has been introduced into the vessel. In the first part of the fermentation, there is a strong decrease of dissolved oxygen

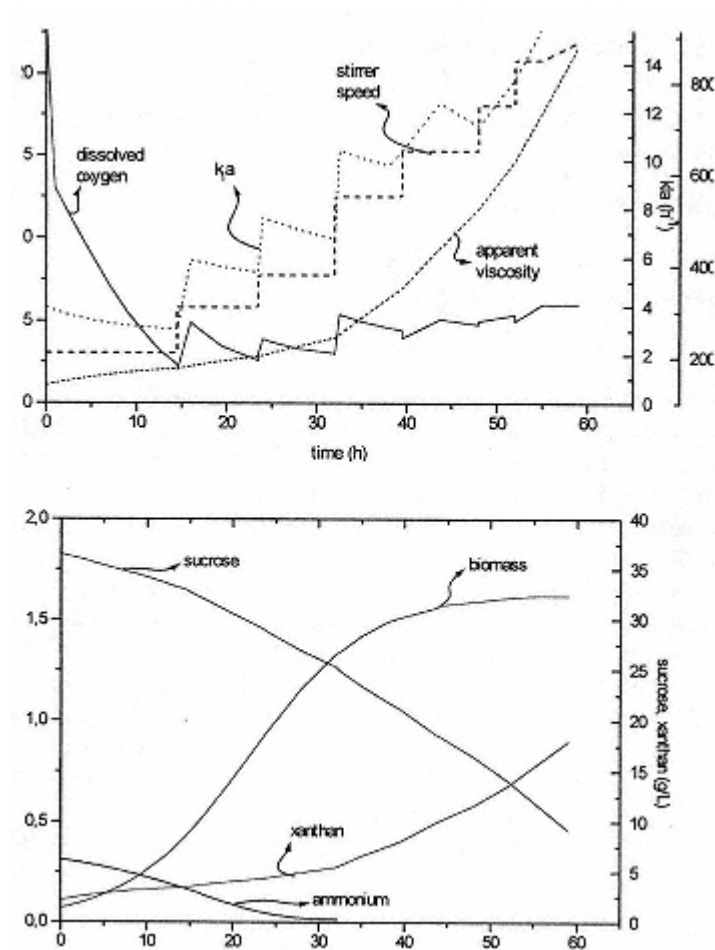


Fig. 4.
Evolution of different compounds and variables during xanthan production. Simulation carried out using a kinetic model given elsewhere (15).

concentration corresponding to the growth of the microorganism; this decrease in dissolved oxygen concentration is faster as inoculum biomass concentration is higher.

2. During the process, the fermentation broth becomes bright yellow with a great increase of its viscosity. The viscosity obtained produces a very important decrease of the oxygen transport rate, and as a consequence a decrease in the dissolved oxygen concentration. For successful production this concentration has to be maintained higher than 10% of saturation value.

3. This must be done by increasing the stirrer speed, with a stirrer speed program during the fermentation time. Fermentations carried out

in a 2-L work volume fermentor usually finish at stirrer speed around 1000 rpm (see Note 3).

Figure 4

gives a typical evolution of the system, showing the concentration evolution of several compounds and variables during xanthan production (15).

3.1.4— **Analytical Methods**

To check the state of the fermentation, some analytical techniques must be used. The main components to be analyzed are: biomass, xanthan, carbon source (sucrose), and nitrogen source (ammonium) concentrations. Dissolved oxygen is also a very important component to consider, but it is usually monitored on line by means of an electrode.

3.1.4.1— **Biomass Analysis**

Biomass concentration is obtained by means of the measurement of the optical density at 540 nm of the diluted broth. Biomass concentration can be determined according to:

$$C_B = 0.2845 \cdot OD_{540 \text{ nm}}$$

where: C_B is biomass concentration (g/L) and $OD_{540 \text{ nm}}$ is optical density at 540 nm.

3.1.4.2— **Xanthan Analysis.**

Xanthan gum concentration can be obtained by dry weight of xanthan isolated by precipitation, but also as a function of broth apparent viscosity. A calibration for each experiment is needed, because the xanthan gum produced is quite different depending on operational conditions employed for production (see **Note 4**). The calibration is made as following: 100 mL of final fermentation broth are used to determine the final xanthan concentration obtained in the production by precipitation (see **Subheading 3.2.**). Other volumes of final broth are diluted and the apparent viscosity of the samples is measured. When the final concentration of the broth is known, the concentrations of the different dilutions made are also known. The results obtained are fitted to know the values of the (A and B) of the following equation:

$$C_X = A \cdot \mu_a^B$$

where C_x is xanthan concentration (g/L) and μ_a is apparent viscosity (cP).

The apparent viscosity values of the samples obtained during fermentation introduced in the expression of the calibration yield the xanthan concentrations during the experiment. The validity of the method can be checked by means of xanthan dry weight determinations at different fermentation times.

3.1.4.3— **Sucrose Analysis**

The best method to determine the concentration of sucrose in samples of xanthan gum production is an enzymatic kit (see **Note 5**).

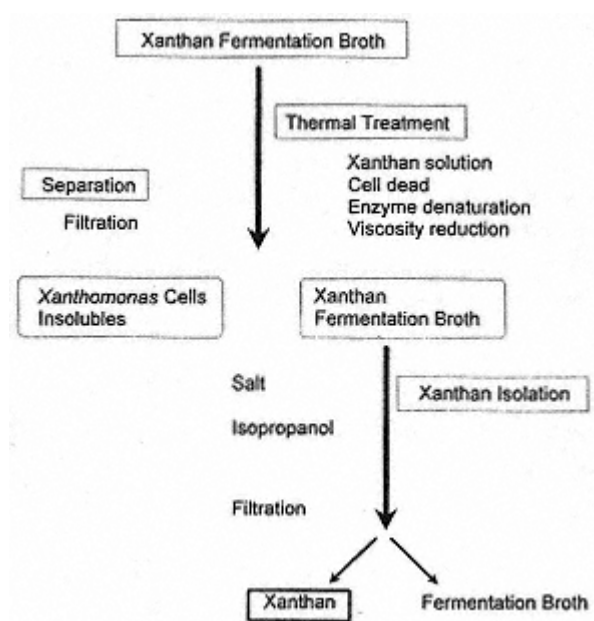


Fig. 5.
Xanthan precipitation scheme.

3.1.4.4—

Ammonium Analysis

A specific electrode (Orion, 95-12 model) is the best method to employ.

3.2—

Isolation of Xanthan Gum

In **Fig. 5**, a diagram of final xanthan isolation process is shown.

1. The first step is to heat the xanthan fermentation broth at 90°C for 15 min. This thermal treatment has different objectives: it enhances xanthan solubility, kills the *Xanthomonas* cells, denatures enzymes that can degrade xanthan, and also decreases broth viscosity.
2. After this treatment and at high temperature, broth is filtered through a 0.45-μm filter. *X. campestris* is 0.4–0.7 μm in width and 0.7–1.8 μm in length (3); therefore, a cake hindering the correct filtration process can quickly be formed. It is necessary to stir the fermentation broth at 2000 rpm to make a suspension and to avoid cake formation (see **Note 6**).
3. Salt is added to the filtered xanthan production broth (free of *Xanthomonas* cells) and dissolved by agitation. Usually 0.5 g/L of NaCl is advised (10) (see **Note 7**).
4. Then IPA is added in enough quantity to produce total polymer precipitation (see **Note 7**). If any cells pass through first filter, alcohol addition can be used to lyse the cells, so they can be eliminated by successive washing.

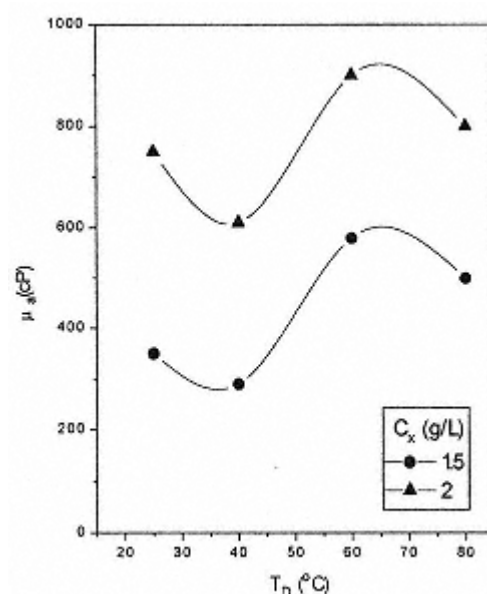


Fig. 6.
Influence of xanthan temperature solution on
solution viscosity.

5. Xanthan precipitate is separated from the broth by a new filtration through a 100- μ m filter. Now broth, lysed cells, salt, and isopropanol pass through the filter while xanthan is retained. IPA may be recovered by distillation.

6. Xanthan obtained is washed with a mixture of IPA-water (approx 3:1 v/v). This process favors cell separation from xanthan gum, and may be repeated until washwater comes out clean (see Note 8).

7. Xanthan can be dried, milled, and packed in any suitable commercial form.

After xanthan is obtained in a stable form, it is necessary to characterize it. Normally, the parameters measured for marketing are: moisture and ash contents, acetate and pyruvate concentration, and viscosity. Moisture and ash content can be measured by thermogravimetry, heating the polymer and registering its loss of weight (see Note 9). Pyruvate and acetate content can be measured using enzymatic kits (see Note 10).

Xanthan shows pseudoplastic behavior, its viscosity in solution changes with shear rate. Xanthan solution viscosity can be determined using a Brookfield viscosimeter. Some variables such as salt and polymer concentrations, solution, and measured temperatures influence this behavior (16). Rheological behavior may be compared only when the viscosity of different xanthan gums has been measured in the same conditions. **Figure 6** shows how xanthan solution viscosity changes with gum solution temperature. Both the Ostwaldde Waele and Casson models have been used in literature to describe xanthan

solution rheological behavior. When the xanthan is dissolved at 25°C solution temperature the following empirical equations can be employed:

$$\mu_a = k \cdot \gamma^{(n-1)}$$

$$k = 215 \cdot C_X^{2.4} \cdot \text{Exp}(-0.0237 \cdot T_M)$$

$$n = 0.558 - 0.154 \cdot C_X + 0.0032 \cdot T_M$$

where μ_a is apparent viscosity (cP); γ is shear rate (s^{-1}); C_x is xanthan concentration (g/L); T_M is measurement temperature ($^{\circ}C$).

4—

Notes

1. The sterilization of YM-T medium must be carried out as follows: The solution containing the carbon source must be sterilized apart from the solution containing salts (both solutions have to be concentrated to obtain the required concentration of nutrients when they are mixed). It is also necessary to sterilize all the Erlenmeyer flasks that are going to be employed and some tips of different pipettes. After sterilization, the carbon source and the other nutrients have to be mixed in a vertical laminar-flow work station and afterwards 43 mL of the sterile medium have to be introduced in the sterile Erlenmeyer flasks.
2. Usually the fermentors have accessories in their tops to allow introduction of medium or different solutions. They have also peristaltic pumps to introduce solutions of alkali, acid or antifoam. The carbon source solution is sterilized connected to the vessel through one of the inputs at its top and when it has been sterilized a peristaltic pump is employed to introduce the sucrose solution into the vessel. The temperature of the medium has to be lower than 60°C. Special care has to be taken closing the unions of the vessel to the carbon source bottle to avoid possible movement of the liquids.
3. When the fermenter size is about 2 L, flat-blade impellers must not be used because microorganisms can be damaged. When a fermenter of larger work volume is used, a test of damage has to be carried out by culturing of *X. campestris* growth with and without flat blades under the same operational conditions.
4. Operational conditions used to carried out xanthan production have a great influence on the characteristics of the final product, i.e., molecular weight and acetate and pyruvate contents in the molecule. As temperature value used for production increases, molecular weight and pyruvate content decreases and acetate content increases. When dissolved oxygen concentration is higher (due to an increase of stirrer speed), the xanthan gum obtained has both higher molecular weight and higher contents of acetate and pyruvate. When the stirrer speed employed is excessively high for the time of fermentation, cells are damaged and xanthan produced has lower molecular weight and contents in acetate and pyruvate, if the cells are able to produce xanthan gum (7).
5. The phenol-sulfuric method (17) cannot be employed in this fermentation to analyze sucrose because xanthan gum interferes in the analysis. This analytical technique has a very high object error.

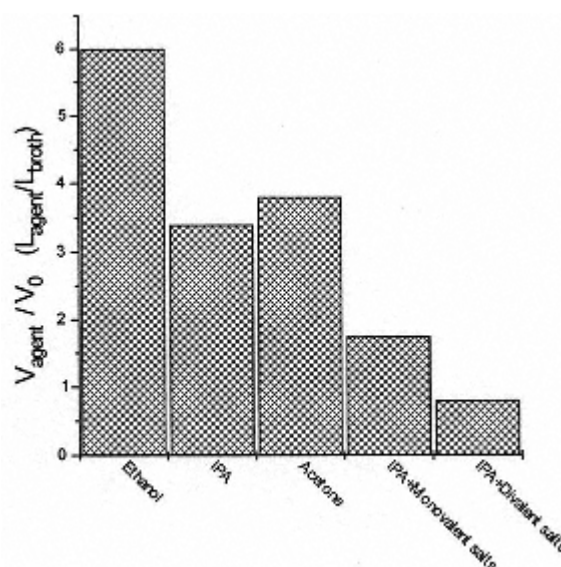


Fig. 7.

Utilization of different agents for xanthan precipitation.

6. To filter xanthan production broth without cell cake formation it is necessary to use a complex assembly. The funnel where the filter is located must be heated at a constant temperature of 75° C. Furthermore, it must be coupled with a helix agitator to maintain cells in suspension and to avoid cell-cake formation. It is necessary to have a pressure difference between broth and filtrate of at least 720 mmHg to achieve a continuous filtration. If this process is not followed, cells will obstruct the filter pores, and it will be necessary to clean the filter. When fermentation broth shows high viscosity, a mixture of IPA-water or IPA alone can be added to ease filtration. Nevertheless, IPA volume should not be greater than $1.3 V_{\text{IPA}}/V_{\text{broth}}$ ratio, otherwise xanthan precipitates helped by salts not consumed in cell growth. Xanthan precipitation can entrap dead cells and intensive washing would be necessary for xanthan recovery.

7. Salt enhances xanthan precipitation, reducing the alcohol concentration (or volume) required in this process (10). This reduction is higher with divalent cations than with monovalent ones, as can be seen in Figs. 7 and 8. The problem when divalent salts are employed is that xanthan precipitates as a salt of low solubility, so it becomes necessary to exchange the divalent cation with another monovalent ion, such as sodium or potassium to obtain xanthan gum with high solubility.

8. *X. campestris* shows yellow color and its presence can be detected by optical density at 540 nm. This property allows the presence of cells in the washing-water to be detected.

9. Xanthan is a hygroscopic product, consequently its moisture content will depend on environmental humidity, so it must be kept in a dry and cool place. Moisture

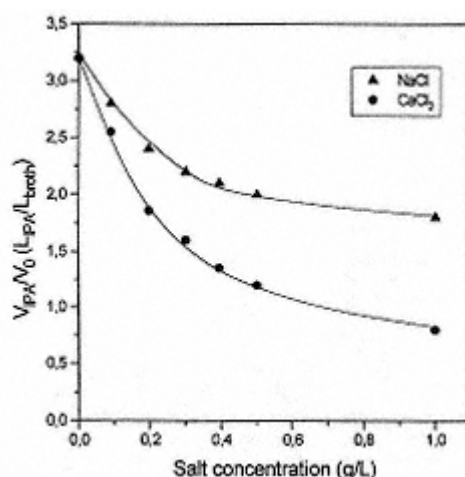


Fig. 8.
Influence of salt addition on IPA volume
necessary for total xanthan precipitation

and ash content can be measured using a Dupont 951 thermogravimetric analyzer with the following conditions: atmosphere of air at 1.25 cm³/s flow rate, 5°C/min heating rate and 15 mg xanthan sample. Typical values measured were 10% (w/w) water and 10% (w/w) ash.

10. Acetate and pyruvate xanthan contents may be measured using commercial kits after xanthan deacetylation and depyruvylation of 1 mL solution with 3–5 g/L of xanthan (**18**). Deacetylation and depyruvylation steps must be carried out as follows:

Deacetylation: 1 mL 0.2 M KOH is added to the xanthan solution sample. Afterwards, the vessel is filled with N₂₃ and heated at 45°C for 6 h. After this, 0.1 M H₃PO₄ is added until acidity and 3 mL distilled water are added before centrifugation at 3000 rpm for 10 min (1600g). The supernatant is used to measure the acetate content using an enzymatic kit (Boehringer-Mannheim no. 14261).

Depyruvylation: 1 mL 0.1 M H₃PO₄ is added to the xanthan solution sample. The vessel is sealed and heated at 90°C for 90 min. Afterwards, 3 mL distilled water is added before centrifugation at 3000 rpm for 10 min. The supernatant is used to measure the pyruvate content by an enzymatic kit (Boehringer-Mannheim, no. 124982).

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3—

Alginate from *Azotobacter vinelandii*

Francesca Clementi, Mauro Moresi, and Eugenio Parente

1—

Introduction

Alginates are a group of polysaccharides occurring as structural components or as capsular materials in the cell wall of the brown seaweeds or soil bacteria, respectively **(1)**. About 30,000 metric tons of sodium alginates per year are currently used in the food, pharmaceutical, textile and paper industries as thickening, stabilising and jellifying agents **(2)**. Since only a few of the many species of brown algae are suitable and are limited in abundance and location for commercial alginate production, there is at present interest in the bacterial production of alginate-like polymers **(3–5)**.

Alginate production from glucose-based media by *Azotobacter vinelandii* DSM 576 was previously optimized at the shaken-flask scale with respect to several operating variables, such as fermentation temperature, shaking speed, glucose concentration, C/N ratio, and sodium phosphate and acetate concentrations in buffered media **(6)**. Then the polymer collected from the culture broth at different fermentation times was characterized by a mannuronate fraction (M) of $73 \pm 2\%$ and classified of the high-mannuronic type **(7)**, similar to the alginates prepared from *Ascophyllum nodosum* and *Macrocystis pyrifera*. Moreover, the proportion of guluronic-guluronic blocks was quite small ($GG = 0.037 \pm 0.006$) and the percentage of acetylated mannuronic units (i.e., acetylation degree) was of the order of $18 \pm 8\%$ **(7)**, both these fractions being typical of native bacterial alginates **(8)**. Finally, whereas the physico-chemical properties of this biopolymer were practically independent of the fermentation time, its average molecular mass (M_M) started to reduce as cell growth had stopped, probably because of the release of alginate lyases **(9)**.

The main aim of this work is to provide detailed practical procedures for monitoring alginate production by *A. vinelandii* in the shaken-flask and laboratory-fermenter scales.

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The reagent for the spectrophotometric determination of alginate is prepared by adding 1.5 mL of Vantocil IB (20% aqueous solution of polymeric biguanidine chloride, PHMBH⁺Cl⁻, available from Zeneca Biocides, PO Box 42, Hexagon House, Manchester M9 8ZS, UK, fax: 44-161-7956005) to 50 cm³ of 2% sodium acetate in a 100-cm³ volumetric flask and then bringing to volume with distilled water, to obtain a final concentration of 0.3% PHMBH⁺Cl⁻ in 1% sodium acetate. This operation should be performed carefully since Vantocil IB can cause irritation to eyes, nose, and respiratory tract. The reagent should be stored in tightly closed polyethylene bottle away from bright light. Although Vantocil IB is stable for 2 yr, the reagent should usually be consumed within a month.

Sodium alginate extracted from *Laminaria hyperborea* (BDH, Poole, UK) is used to construct the calibration curve of the spectrophotometric method.

2.6—

Residual Glucose Assay

The residual glucose (S) in the supernatant is determined by using the dinitrosalicylic acid (DNSA) method (11). To prepare the specific reagent of this method, it is necessary to dissolve 10 g of 3,5-dinitrosalicylic acid, 2 g of phenol, 0.5 g of sodium sulfite, and 200 g of potassium sodium tartrate in 500 cm³ of 2% (w/v) NaOH and bring the solution to 1 dm³ in a volumetric flask by adding distilled water. The reagent is quite stable, its sensitivity being practically unaltered within 3 mo.

2.7—

Residual Ammoniacal Nitrogen Assay

The residual ammoniacal nitrogen (N) in the supernatant is determined using a modified version of Strickland and Parson's method (12). To prepare its specific reagents, the following procedure is used:

1. Dissolve 140 g of trisodium citrate and 5 g of sodium hydroxide in distilled water using a 1-dm³ volumetric flask. The reagent is stable at room temperature.
2. Dissolve 35 g of phenol and 0.40 g of sodium nitroprusside in 900 cm³ of distilled water and bring to volume using a 1-dm³ volumetric flask. Let the solution stand for about 17 h before using it. The reagent is stable for 3 wk if stored in a dark bottle at 4°C.
3. Dissolve 1 g of dichloroisocyanuric acid sodium salt and 10 g of sodium hydroxide into 100 cm³ of distilled water and bring to volume using a 500-cm³ volumetric flask. Let the solution stand for about 17 h before using it. The reagent is stable for one wk if stored in a dark bottle at 4°C.

Warning: Reagents 2. and 3. are highly toxic.

2.8—

Intrinsic Viscosity Estimation

Cannon-Fenske capillary viscometers with size numbers ranging from 50 to 150 (corresponding to capillary tubes with inside diameters varying from 0.42

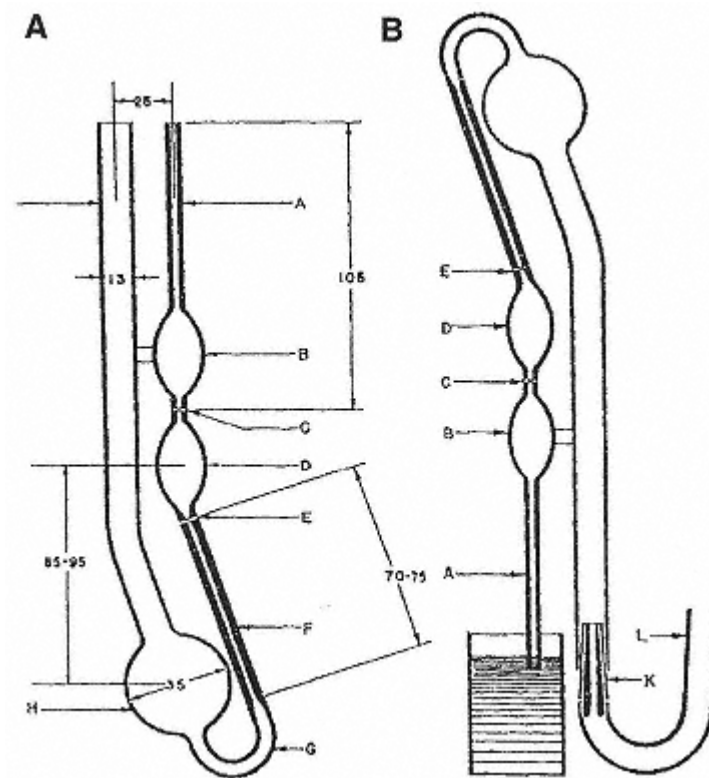


Fig. 1.
Cannon-Fenske capillary viscometer
(reproduced with permission from **ref. 13**).
(A) details of its design and construction with all
dimensions in millimeters. (B) method of introducing sample into
viscometer.

to 0.78 mm) (**13**), and 25-cm³ calibrated volumetric flasks are used for the determination of kinematic viscosity (ν) and density (ρ) on 0.1 M NaCl dispersions containing 0–1.5% (w/v) of bacterial alginate. **Figure 1A** shows some details of design and construction of this viscometer. A Plexiglas container suitable for immersion of the viscometers (so that the liquid reservoir or the top of the capillary is at least 2.0 cm below the upper bath level) is used in order to provide visibility of the viscometers and the thermometer. A firm support for each viscometer is to be provided. The bath is to be filled with distilled water and the bath temperature must be thermostatically regulated by means of a heater and a stirrer so as to keep its variation within $\pm 0.01^\circ\text{C}$ over the length of the viscometer, or from viscometer to viscometer in the various bath positions for temperature above 15.5°C . A stop watch with accuracy of 0.2 s or less is used to measure the efflux time. Double-distilled water is used to calibrate the viscometers used.

3—

Methods

3.1—

Inoculum Buildup and Culture Conditions.

1. 1 cm³ of the frozen stock is transferred to 100-cm³ Erlenmeyer flasks containing 27 cm³ of sterile medium. The seed culture is incubated on a rotary shaker at 300 min⁻¹ and 35°C for 48 h.
2. A fraction (1.5 cm³) of such a culture is used to inoculate a 250-cm³ baffled Erlenmeyer flask containing 50 cm³ of presterilized medium so as to obtain an initial optical density (OD) of 0.4 at 600 nm (9).
3. After growing the seed culture at 120 min⁻¹ and 35°C for 24 h, the broth is used to inoculate the production medium either in the shaken-flasks or in the laboratory-fermenter at OD = 0.4.
4. At the laboratory-fermenter scale, the fermentation process is performed under the following operating conditions: inoculation ratio (ca. 3% v/v), stirrer speed ($n = 400 \text{ min}^{-1}$), temperature (35°C), pH (7.0), and air flow ($Q_A = 0.6 \text{ dm}^3/\text{min}$), and pressure on the tank-top ($P_L = 1.2 \text{ bar}$). Continuous control of pH is achieved automatically to within 0.1 pH unit by adding 6 M NaOH using a peristaltic pump, while foaming is controlled automatically by adding a 10% (v/v) solution of silicon-based antifoam reagent (Antifoam A, Fluka Chimica, Milan, Italy).

3.2—

Separation of Cell Biomass

Separation of *A. vinelandii* cells from the culture broth is achieved by:

1. Adding 0.2-cm³ 5 M NaCl and 0.2-cm³ 0.5 M Na₄EDTA to 10-cm³ sample into dried, pre-weighed tubes.
2. Centrifuging the mixture at 12,000g at 15°C for 20 min.
3. Removing the supernatant.
4. Suspending the residue in 10 mM Na₄EDTA for 2 min to dissolve the cell-associated alginate.
5. Centrifuging as in **step 2**.
6. Removing the supernatant, washing twice the alginate-free cell precipitate with distilled water, and centrifuging.
7. Drying the final residue in an oven at 105°C for 24 h (9) and weighing the tubes to estimate the biomass concentration (X).

3.3—

Alginate Determination

Alginate can be measured either gravimetrically or spectrophotometrically, as shown below.

3.3.1—

Gravimetric Method

Alginate can be precipitated from culture broths by adding several solvents, such as ethanol, isopropanol, and acetone. Once the precipitate has been washed, it can be dried and gravimetrically determined. In this work, the following procedure is used:

2—

Materials

2.1—

Microorganism.

Azotobacter vinelandii DSM 576 is used. Lyophilized cultures are obtained from Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany). The stock culture is grown on Tryptic Soy Broth (TSB) (Difco, Detroit, MI) integrated with 0.6% (w/v) of yeast extract (Difco, Detroit, MI) for 24 h at 35°C and then maintained as frozen stock in 25% glycerol at -75°C.

2.2—

Seed and Production Media

The seed and production media contain per liter of distilled water: 20 g glucose, 0.6 g $(\text{NH}_4)_2\text{SO}_4$, 2 g Na_2HPO_4 , 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 6 g yeast extract (**10**). The seed and production media in the shaken-flasks are buffered at about 7.2 by adding 11.56 g of 3(*N*-morpholino)-propane-sulfonic acid monosodium salt (MOPS-Na). In the laboratory-fermenter scale no addition of MOPS-Na is used, the pH of the production medium being automatically controlled by on-demand addition of 6 M NaOH. All the reagents used are of analytical grade. Before use, all media are sterilized at 121°C for 15 min (**6**).

2.3—

Experimental Rigs

A rotary shaker is used to study the fermentation process at the shaken-flask scale, and a 3-dL stirred-tank fermenter STF (Applikon, Schiedam, NL) is used for performing the laboratory-scale experimentation. The STF consists of a cylindrical vessel (130-mm internal diameter and 250-mm height), equipped with two 6-flat-bladed turbines (diameter 45 mm) and 3 baffles (13-mm width), automatic control of stirrer speed (n), temperature (T), pH, antifoam, and dissolved oxygen concentration (DO). The fermenter is filled with the production medium and sterilized at 121°C for 15 min.

2.4—

Separation of Cell Biomass

Five molar NaCl and 0.5 M tetrasodium ethylenediamine tetraacetic acid (Na_4EDTA) are added to each sample of the culture broth tested before centrifugation. Ten millimolar Na_4EDTA are used to remove soluble nitrogen and cell-associated alginate from the solid residue containing bacteria and capsular material.

2.5—

Alginate Recovery and Determination

The exopolysaccharide is precipitated from the alginate-containing supernatants obtained as in **Subheading 2.4**. by adding 95% (v/v) ethanol at -20°C, and then dehydrated using pure ethanol in the case of its further processing to assess its average molecular mass or chemical composition, as described in **Subheading 4.3.1**.

The reagent for the spectrophotometric determination of alginate is prepared by adding 1.5 mL of Vantocil IB (20% aqueous solution of polymeric biguanidine chloride, PHMBH⁺Cl⁻, available from Zeneca Biocides, PO Box 42, Hexagon House, Manchester M9 8ZS, UK, fax: 44-161-7956005) to 50 cm³ of 2% sodium acetate in a 100-cm³ volumetric flask and then bringing to volume with distilled water, to obtain a final concentration of 0.3% PHMBH⁺Cl⁻ in 1% sodium acetate. This operation should be performed carefully since Vantocil IB can cause irritation to eyes, nose, and respiratory tract. The reagent should be stored in tightly closed polyethylene bottle away from bright light. Although Vantocil IB is stable for 2 yr, the reagent should usually be consumed within a month.

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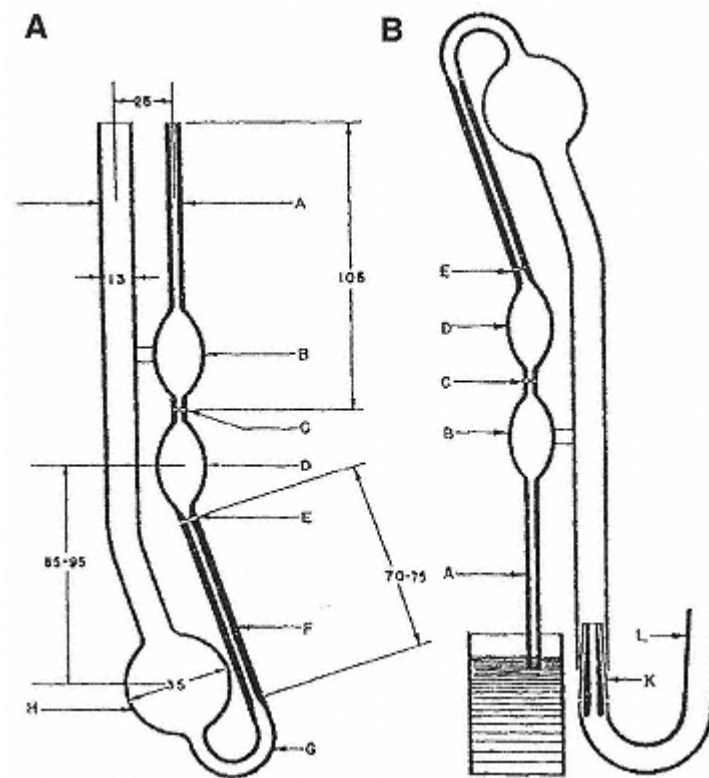


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2. Centrifuging the mixture at 12,000g at 15°C for 20 min.
3. Removing the supernatant.
4. Suspending the residue in 10 mM Na₄EDTA for 2 min to dissolve the cell-associated alginate.
5. Centrifuging as in **step 2**.
6. Removing the supernatant, washing twice the alginate-free cell precipitate with distilled water, and centrifuging.
7. Drying the final residue in an oven at 105°C for 24 h (9) and weighing the tubes to estimate the biomass concentration (X).

3.3—

Alginate Determination

Alginate can be measured either gravimetrically or spectrophotometrically, as shown below.

3.3.1—

Gravimetric Method

Alginate can be precipitated from culture broths by adding several solvents, such as ethanol, isopropanol, and acetone. Once the precipitate has been washed, it can be dried and gravimetrically determined. In this work, the following procedure is used:

1. Add 3 vol of ice-cold 95% ethanol to 1 vol of culture broth at 4°C into preweighed tubes, stirring slowly.
2. Centrifuge the mixture at 12,000g at 4°C for 10 min.
3. Remove the supernatant carefully.
4. Resuspend the gel-like residue in 1 vol of distilled water.
5. Add 3 vol of ice-cold 95% ethanol, stirring slowly.
6. Centrifuge the new mixture in pre-weighed tubes at 12,000g at 4°C for 10 min.
7. Remove the supernatant carefully.
8. Dry the tubes in the oven at 105°C for 24 h and determine the alginate dry weight and concentration (P).

3.3.2—

Spectrophotometric Method

The only satisfactory spectrophotometric method available for alginate measurement was originally developed by Kennedy and Bradshaw (14) to assay alginate from brown seaweeds and, subsequently, modified to measure bacterial alginates (3,15). The following procedure, adapted from Brivonese and Sutherland (3), is used:

1. Add 1 cm³ of 0.3% PHMBH⁺Cl⁻ in 1% sodium acetate to 0.5 cm³ of culture supernatant (eventually diluted to obtain a final alginate concentration less than 3 g.dm⁻³) into 1.5-cm³ Eppendorf microcentrifuge tubes.
2. Stir the mixture for 5 min by intermittent vortexing (warning: it is essential to standardize the agitation procedure [14]).
3. Centrifuge at 12,000g for 5 min to pelletize the PHMBH⁺Cl⁻-alginate complex.
4. Dilute the supernatant 1:50 in distilled water.
5. Read the optical density of sample and blank (the latter being prepared using distilled water in place of the supernatant) at 235 nm against distilled water using quartz cuvetts.
6. Calculate the alginate concentration (P, in g.dm⁻³) using Eq. 1:

$$P = a_p [OD_B - OD] = a \Delta OD_{235} \quad (1)$$

where a_p ($=3.25 \pm 0.03$, $r^2 = 0.992$) is an empirical coefficient, estimated by using the least squares method. Such an equation was determined by plotting any prefixed alginate concentration (P) vs the difference between the optical density of a blank (OD_B) and that of a generic calibration sample (OD), prepared by suspending a given amount of sodium alginate from *L. hyperborea* (BDH, Poole, UK). Once 4 g of this alginate was dissolved into 1 dm³ of distilled water, a series calibration samples containing 0.4, 0.8, 2.0, and 3.2 g.dm⁻³ was obtained via further dilution. Each sample was then submitted to **steps 1–5** to determine the calibration curve shown in **Fig. 2**.

3.4—

Residual Glucose Assay

The DNSA method (**11**) used to assess the residual glucose (S) in the supernatant is performed as follows:

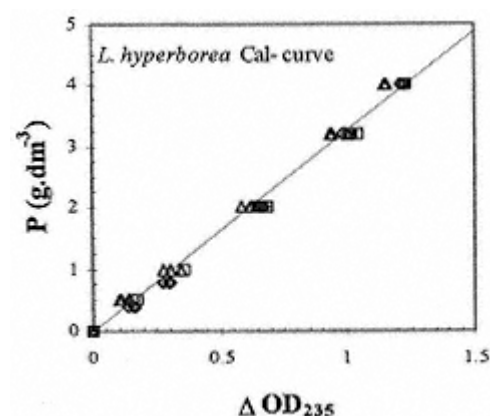


Fig. 2.

Calibration curve for alginate determination using the spectrophotometric method: Alginate concentration (P) vs the difference of optical density (ΔOD_{235}) at 235 nm between a blank and the calibration sample containing sodium alginate from *L. hyperborea* (BDH). The symbols used refer to three replicated calibration experiments.

1. Mix 2 cm³ of the supernatant with 3 cm³ of the DNSA reagent using 16 × 160-mm test-tubes.
2. Place the test-tubes into a boiling water bath for 15 min.
3. Let the tubes cool into an ice-slush for 10 min and then keep them at room temperature for 5 min.
4. Transfer the tube content into disposable macrocuvets.
5. Read their optical density at 640 nm against a blank prepared according to the same procedure by replacing the supernatant with distilled water.
6. Calculate the glucose concentration (S , in g.dm⁻³) using **Eq. 2**:

$$S = a_s OD_{640} \quad (2)$$

where a_s ($=0.99 \pm 0.02$, $r^2 = 0.994$) is an empirical coefficient, estimated by using the least squares method. Such an equation was determined by plotting any prefixed glucose concentration (S) against the optical density (OD_{640}) of a calibration sample, prepared by dissolving a given amount of oven-dried anhydrous glucose. Once 1 g of glucose was dissolved into 1 dm³ of distilled water, the mother solution was further diluted to get 6 calibration samples containing 0.1 to 0.6 g.dm⁻³. Each of these samples was then submitted to **steps 1–5** to determine the calibration curve shown in **Fig. 3**.

3.5—

Residual Ammoniacal Nitrogen Assay

To assess the residual ammoniacal nitrogen (N) in the supernatant, the Strickland and Parson method (12) is carried out as follows :

1. In a disposable macrocuvet, add, in order, 50 μ L of the supernatant and the reagents described in **Subheading 2.7.**, 1-cm³ each.

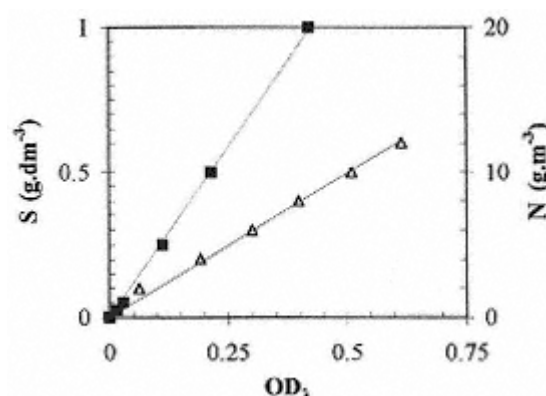


Fig. 3.
Calibration curve for glucose (S) and ammoniacal nitrogen (N) determination using the DNSA (11) and Strickland and Parson (12) methods, respectively. Glucose (Δ) and ammoniacal nitrogen (■) concentrations against the optical density (OD_λ) at 640 and 630 nm of calibration samples.

2. After 1 or 2 h incubation in the dark read the optical density at 630 nm against a blank prepared with distilled water.

3. Calculate the ammoniacal nitrogen concentration (N , in g.dm⁻³) using **Eq. 3**:

$$N = a_N OD_{630} \quad (3)$$

where $a_N (=47.2 \pm 0.5, r^2 = 0.999)$ is an empirical coefficient, estimated by using the least squares method. Such an equation was determined by plotting any prefixed ammoniacal nitrogen concentration (N) vs the optical density (OD_{630}) of a calibration sample, prepared by dissolving a given amount of ammonium chloride. Once 0.3819 g of ammonium chloride were dissolved into 1 dm³ of distilled water, the resulting mother solution containing 100 g.m⁻³ of nitrogen was appropriately diluted to obtain at least 5 calibration samples in the range of 0.5–20 g.m⁻³. Each sample was then submitted to **steps 1–2** to determine the calibration curve shown in **Fig. 3**.

3.6—

Intrinsic Viscosity Estimation

The intrinsic viscosity [μ] is a characteristic property of a single macromolecule in a given solvent and is a measure of the hydrodynamic volume occupied by the polymer itself. It depends primarily on molecular mass (M_M), chain rigidity and solvent quality.

Smidsrød (16) demonstrated that the intrinsic viscosity of different alginates at infinite ionic strength was independent of the counter-ion used (Li^+ , Na^+ , K^+ , Mg^{2+}) and representative of the uncharged alginate molecule, being also identical to that obtained for the alginic acid form in 0.1 M HCl, when the macromolecule contains no charged groups. In this way, the intrinsic viscosity

is usually correlated to the average molecular mass (M_M) via the well-known Mark-Houwink equation (**Eq. 4**) (**17**):

$$[\mu] = K (M_M)^a \quad (4)$$

where K and a are empirical constants.

To determine the intrinsic viscosity of the bacterial alginate the following procedure is used:

1. Determine the kinematic viscosity (ν) of 0.1 M NaCl dispersions containing 0–1.5% (w/v) of bacterial alginate at 25°C using the following standard procedure D-445-61 of the American Society for Testing and Materials (**13**):

- a. Maintain the water bath at the test temperature (25°C) within $\pm 0.01^\circ\text{C}$.
- b. Filter a portion of the sample through a 200-mesh screen.
- c. Select a clean, dry no. 50–150 Cannon-Fenske capillary viscometer (**Fig. 1A**), which will give an efflux time not shorter than 200 s.
- d. Fill the viscometer as shown in **Fig. 1B** by inverting the viscometer and applying suction to tube L to cause the sample to rise through bulbs B and D to etched line E. Once the viscometer has been returned to its normal vertical position, wipe tube A clean, insert the viscometer into a holder and placed it into the constant-temperature water bath.
- e. Allow approx 5 min for the viscometer to reach the bath temperature.
- f. Align the viscometer vertically in the bath by using a plumb bob in tube J (**Fig. 1A**).
- g. Apply suction to tube A and bring sample into bulb D, about 5 mm above mark C.
- h. Measure in seconds, to within 0.1 s, the time required for the meniscus to pass from the first timing mark (C) to the second (E). If the efflux time is less than 200 s, select a smaller capillary viscometer and repeat the operation.
- i. Repeat the procedure described in paragraphs **steps 1a-1h**, making at least three determinations of the efflux time (t).
- j. Estimate the mean value (t_m) and corresponding standard deviation (t_{sd}) of the efflux time, both expressed in seconds.
- k. Use the above data for calculating the mean value (ν_m) and corresponding standard deviation (ν_{sd}) of the kinematic viscosity (ν) of any sample as shown in **Eq. 5**:

$$\nu_i = C t_i$$

where C is the calibration constant (in centistokes per second) of the capillary viscometer used and ν_i is expressed in centistokes (cS).

2. Measure the density (ρ) of all dispersions examined by using clean, dry, preweighed 25-cm³ pycnometers. Once filled each calibrated volumetric flask with the sample at 25°C up to top-flask level, the filled flask should be stored in a thermostat at 25°C for about 30 min and then weighed. The difference between the masses of the full and empty flasks, expressed in grams, divided by the volume of the calibrated flask, expressed in cm³, yields the density (ρ) of the sample, expressed in g.cm⁻³.

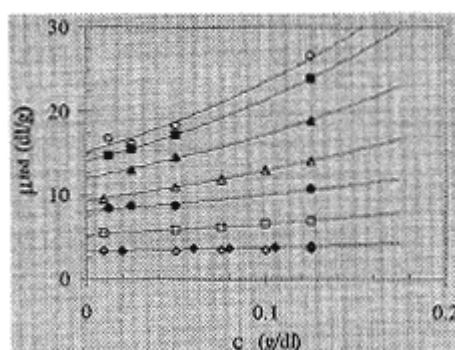


Fig. 4.

Effect of alginate concentration (c) on the reduced viscosity (μ_{red}) at 298.16 degrees Kelvin (K) of 0.1 M NaCl dispersions of a range of biopolymers produced by *A. vinelandii* DSM 576 characterized by different values of the average molecular mass (M , in kDa): $\blacklozenge$, 39 ; $\diamond$, 40, $\square$, 64 ; $\bullet$, 101 ; $\triangle$, 116; $\blacktriangle$, 151; $\blacktriangle$, 178 ; $\circ$, 193.

3. Calculate the dynamic viscosity (μ) of the sample as shown in **Eq. 6**:

$$\mu = \eta \rho \quad (6)$$

where μ is expressed in centipoise (cP) or millipascal per second (mPa.s).

4. Estimate the reduced viscosity (μ_{red}) as suggested by Launey et al. (17):

$$\mu_{\text{red}} = (\mu - \mu_s) / (\mu_s c) \quad (7)$$

where μ_s is the viscosity of the solvent and c the alginate concentration, expressed in g/dL.

5. Estimate the intrinsic viscosity $[\mu]$ by extrapolating the plot of the reduced viscosity vs c for c tending to 0:

$$[\mu] = \lim_{(c \rightarrow 0)} \mu_{\text{red}} \quad (8)$$

6. Since some $\mu_{\text{red}} - c$ relationships are nonlinear, as already revealed by Donnan and Rose (18) and confirmed by the diagram in **Fig. 4**, correlate the $\mu_{\text{red}} - c$ data using **Eq. 9**:

$$\mu_{\text{red}} = [\mu] \exp (\delta c) \quad (9)$$

where δ is an empirical coefficient, estimated by the least squares method.

7. Predict the average molecular mass (M_M) of the bacterial alginates under study using the Mark-Houwink regression derived from Donnan and Rose's experiments (18), that were referred to a series of alginates extracted from *Laminaria cloustoni*:

$$M_M = 1.151 \times 10^4 [\mu]^{1.04} (r^2 = 0.96) \quad (10)$$

where $[\mu]$ and M_M are expressed in dL/g and g/mol or Daltons, respectively. It can be noted that the above exponent falls within the range of values (0.92–1.13),

previously estimated by Martinsen et al. (19) and Smidsrød (16) for several other algal alginates.

4—

Notes

4.1—

Production Media and Culture Conditions.

Alginate production by *A. vinelandii* is deeply affected by the strain, production medium, incubation conditions, and fermentation equipment used, as summarized in **Table 1 (3,9,20–24)**.

In previous work (6), alginate production from glucose-based media by *A. vinelandii* DSM 576 was found to be highly sensitive to pH reduction. Once the culture medium was buffered with MOPS-Na, alginate secretion was hampered by sodium acetate addition, being insensitive to phosphate concentrations and C/N ratios in the ranges of 0.035–1.8 g.dm⁻³ and 60–110 g-atom C per g-atom N, respectively (6). The importance of phosphate limitation was already pointed out by several authors, such as Horan et al. (23). However, the fact that alginate yields as high as 25% of the glucose supplied were achieved at high levels of phosphate (3.5 g.dm⁻³) and acetate (3 g.dm⁻³) (6) confirmed the so called optimal results obtained by Brivonese and Sutherland (3) when using phosphate- and nitrogen-rich media.

Although Jarman et al. (25) stated that pH-controlled alginate production by *A. vinelandii* is practically unaffected by most environmental conditions, data from literature, as well the present authors' experience in batch fermentations using different substrates under different DO concentrations (20), show that key variables for bacterial alginate production have not been identified yet or are highly strain-dependent. Therefore, any eventual industrial-scale production of microbial alginate asks for further work to assess alternative very low-cost synthetic production media.

Whatever the culture collection or mutant strain used, batch alginate production seem to be almost similar and of the order of 6 g.dm⁻³ with alginate yield factors on dry cell ($Y_{p/X}$) and sugar consumed ($Y_{p/S}$) of about 0.4 g.g⁻¹ and 0.15–0.38 g.g⁻¹, respectively. Continuous alginate production resulted in a yield as great as one g of alginate per g of sugar consumed (24).

Owing to the pseudoplastic behavior of alginate in aqueous dispersions (1,9), the oxygen transfer capability of the fermentation vessel used should affect the bacterial productivity, as confirmed by a series of fermentation runs carried out in the laboratory fermenter described in this work at different levels of the dissolved oxygen concentration in the culture broth (20).

Finally, by performing the fermentation process at the shaken-flask scale as described in **Subheading 3.1.**, it is possible to replicate not only the alginate production process, but also its polymerization degree with the degree of precision shown in **Fig. 5**.

Table 1**Composition of Production Media, Microbial Strains, and Culture Conditions Used to Perform Alginate Production by *Azotobacter vinelandii***

<i>Production Medium</i>	<i>Strain</i>	<i>Culture Conditions</i>	<i>Yield</i>	<i>Reference</i>
A. As the production medium used in this work (10)	DSM 576	SF $T = 35\text{ }^{\circ}\text{C}$ $n = 300\text{ rpm}$ $\text{pH} \approx 7.2$ $t = 48\text{ h}$	$P = 5.7\text{ g. dm}^{-3}$ $Y_{\text{p/X}} = 0.31\text{ g/g}$ $Y_{\text{p/X}} = 0.40\text{ g/g}$	(9)
B. As A without MOPS-Na	DSM 576	2.5-dm ³ batch STF $T = 35\text{ }^{\circ}\text{C}$ $n = 400\text{ rpm}$ $\text{pH} = 7.2$ $t = 72\text{ h}$	$P = 3.9\text{ g. dm}^{-3}$ $Y_{\text{p/S}} = 0.22\text{ g/g}$ $Y_{\text{p/X}} = 0.41\text{ g/g}$	(21)
C. Glucose 40 g NH_4NO_3 1.5 g KH_2PO_4 7.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2 g CaCl_2 0.33 g PSMDP 4 g Distilled water 1 dm ³	AX	SF $T = 30\text{ }^{\circ}\text{C}$ $n = 280\text{ rpm}$ pH not reported $t = 72\text{ h}$	$P = 6.0\text{ g. dm}^{-3}$ $Y_{\text{p/S}} = 0.15\text{ g/g}$	(3)
D. Sucrose 20 g K_2HPO_4 0.64 g KH_2PO_4 0.16 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2 g NaCl 0.2 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 43 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 15 mg NaMoO ₄ 2.1 mg Distilled water 1dm ³	E	SF $T = 30^{\circ}\text{C}$ $n = 140\text{ rpm}$ $t = 72\text{ h}$ $\text{pH} = 7.4$	$P = 1\text{ g. dm}^{-3}$	(22)
E. Sucrose 20 g Sodium acetate 2 g K_2HPO_4 1.2 g KH_2PO_4 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ 0.15 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.15 mg NaMoO ₄ 0.75 mg Distilled water 1 dm ₃	NCIB 9068 (mutant)	SF $T = 34\text{ }^{\circ}\text{C}$ $n = 170\text{ rpm}$ $t = 96\text{ h}$ $\text{pH} = 6.5$	$P = 6.2\text{ g. dm}^{-3}$ $Y_{\text{p/S}} = 0.33\text{ g/g}$	(23)
F. Sucrose 20 g K_2HPO_4 32 mg KH_2PO_4 8 g	NCIB 9068 (mutant)	SF $T = 30\text{ }^{\circ}\text{C}$ $n = 200\text{ rpm}$	$P = 6\text{ g. dm}^{-3}$ $Y_{\text{p/S}} = 0.38\text{ g/g}$	(24)

(Table continued on next page)

Table 1 (continued)

<i>Production Medium</i>	<i>Strain</i>	<i>Culture Conditions</i>	<i>Yield</i>	<i>Re</i>
MgSO ₄ ·7H ₂ O 0.2 g NaCl 0.2 g CaCl ₂ ·H ₂ O 42 g FeSO ₄ ·7H ₂ O 0.3 mg NaMoO ₄ 1 mg H ₃ BO ₄ 2.9 mg CoSO ₄ ·7H ₂ O 1.2 mg CuSO ₄ ·7H ₂ O 0.1 mg MnCl ₂ ·4H ₂ O 0.09 mg ZnSO ₄ ·7H ₂ O 1.2 mg MOPS 10.5 g Distilled water 1 dm ³	SM 52B)	<i>t</i> = 48–72 h pH = 7.2		
G. As F without MOPS-Na	NCIB 9068 (mutant SM 52B)	2.5-dm ³ continuous STF <i>T</i> = 35 °C <i>n</i> = 1250 rpm <i>t</i> = 48–72 h pH = 7.2 DO = 1	Y _{P/S} = 1 g/g	(24

Abbreviations: PSMDP, papaic soybean meal digest phytone; SF, shaken-flask; STF, stirred-tank fermenter; P, final or maximum alginate concentration in the culture broth; Y_{P/S}, yield factor of alginate on sugar; Y_{P/X}, yield factor of alginate on dry cell.

Under the operating conditions described, bacteria started growing exponentially without any induction phase, but alginate excretion began with 6- to 10-h delay and continued during the declining and stationary cell growth phases (**Fig. 5A**). The average molecular mass (M_M) of the biopolymer paralleled the exponential cell growth phase (**Fig. 5B**), thus showing a remarkable increase up to ca. 220 kDa at the onset ($t = 40\text{--}42$ h) of the cell stationary phase. This practically coincided with the reduction of the ammoniacal nitrogen content (N) in the production medium to less than 10 g.m⁻³ (**Fig. 5B**). From then onward up to 62 h, cell growth halted with finite glucose levels remaining (2–5 g.dm⁻³), whereas alginate production continued and its concentration (P) in the culture broth kept on increasing from about 3 to 5.7 g.dm⁻³. Simultaneously, the M_M of this biopolymer exhibited a drastic reduction from ca. 220 to 10 kDa in as short as 20–22 h, probably because of the release of alginate lyases as cell growth stopped (**Fig. 5B**).

In the laboratory-fermenter scale experiments based on medium B (**Table 1**), the alginate fermentation exhibited almost similar kinetic patterns with practi-

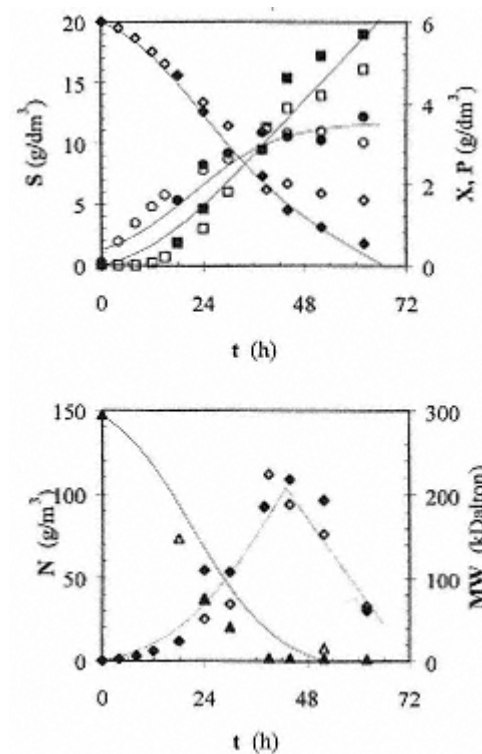


Fig. 5.

Time-course of two replicated A (open symbols) and B (closed symbols) alginate production processes by *A. vinelandii* DSM 576 in the shaken-flask scale: (A)

Concentrations of glucose (S: $\diamond$, $\blacklozenge$), biomass (X: $\circ$, $\bullet$), and alginate (P: $\square$, $\blacksquare$) vs fermentation time (t). (B) Concentration of ammoniacal nitrogen (N: Δ , $\blacktriangle$) and average molecular weight (MW: $\diamond$, $\blacklozenge$) vs t.

cally constant alginate yields per unit dry cell mass ($Y_{p/X}$), but with a 30% reduction in the alginate yield per unit glucose consumed ($Y_{p/S}$), as shown in **Table 1**

Further work should be direct to maximize alginate yield per unit glucose consumed in the laboratory-fermenter scale, as a prerequisite to assess the economic benefits of this fermentation process.

4.2—

Separation of Cell Biomass

High concentrations of alginate in fermentation broths increase broth viscosity and hamper separation of biomass. When dealing with alginate concentrations of 1–2 g.dm⁻³, direct centrifugation of the culture broth at 12,000_g at 15°C for 20 min is generally sufficient to recover the cells. At higher alginate concentrations or at lower temperatures, broth dilution with distilled water up to a ratio of 1:4 is usually necessary before centrifuging.

Alternative procedures are reported in the literature and may involve the following:

1. Filter the supernatant (eventually diluted in 50 mM PIPES/NaOH buffer, pH 8.0, in the case of viscous broths) through 0.7- μm Whatman GF/F filters (23).
2. Dilute 20 g of supernatant to 100 cm^3 with distilled water, then add 6 cm^3 of Na_2EDTA 20%, and centrifuge at 45,000g for 30 min (22).
3. Centrifuge the supernatant at 45,000g for 30 min (26).
4. Add NaCl and Na_4EDTA to the supernatant to obtain the final concentrations of 100 and 10 mM, respectively, and centrifuge at 10,000g at 4°C for 10 min (27).

In a series of replicated determinations of biomass concentration (X) the coefficient of variation (CV, that is the ratio between the standard deviation and the mean value) ranged from 3.2 to 13% with an average value of ca. 8%. However, CV was higher than 10% when X was smaller than 1.5 $\text{g}\cdot\text{dm}^{-3}$.

4.3—

Alginate Determination

4.3.1—

Gravimetric Method

The washing steps 4–6 of the procedure described in **Subheading 3.3.1.** might be omitted, but this would result in an overestimation of the alginate concentration, especially in rich media because of precipitation of salts and peptones. Even in the best conditions, this method lacks sensitivity, since the minimum alginate concentration that can be reliably assessed is 0.1–0.5 $\text{g}\cdot\text{dm}^{-3}$, and has limited accuracy and precision, CV varying from 5 to 20%, depending on type of production medium used. Moreover, some unpredictable overestimation of alginate concentration is to be expected. In fact, in several trials the experimental gravimetric determination of calibrated samples containing algal alginates exceeded the original concentrations by 0.1–0.6 $\text{g}\cdot\text{dm}^{-3}$.

Among the alternative procedures reported in the literature, the following can be cited:

1. Add 3 vol of ice-cold isopropanol to 1 vol of supernatant, stirring slowly; let the mixture stand for 10 min, filter it through preweighed 1.6- μm Whatman GF/A filters, and dry the residue under vacuum at 45°C for 24 h (25,10).
2. Add 1 cm^3 of NaCl 20% to 20 g of turbid culture broth, precipitate the alginate by adding 40 cm^3 of isopropanol, centrifuge at 45,000g for 30 min, and dry in the oven at 105°C to constant weight; estimate alginate weight by subtracting cell dry weight (22).
3. Precipitate alginate from clear culture broths with 2 vol of ice-cold isopropanol; filter the mixture through pre-weighed Gelman A-E filters, and dry the residue (26).
4. Precipitate the alginate from the supernatant with 3 vol of isopropanol, wash the residue with a mixture of isopropanol and water in the ratio 3:1 and dry it at 80°C till constant weight (27).

All the aforementioned critical aspects of the ethanol-based gravimetric method stand for the alternative procedures given above.

Any further processing of the alginate in order to determine its average molecular mass or chemical composition requires further purification steps by:

1. Adding one volume of absolute ethanol at room temperature to dehydrate the polymer recovered.
2. Centrifuging the mixture at 12,000g at 4°C for 10 min.
3. Removing carefully the supernatant and evaporating the residual ethanol under vacuum at temperature less than 60°C.

Evaporation under vacuum results in a very tough, rubbery material which dissolves very slowly. To improve purification, the precipitate from the washing step (i, above) might be dissolved in 0.05 M NaCl and, using Visking tubing, dialysed against 10 vol of 0.05 M NaCl (to remove impurities) and finally against distilled or deionized water with several changes. The alginate can then be reprecipitated and evaporated or freeze-dried. Removal of residual moisture is sometimes difficult, and purified samples should be stored in a desiccator containing P₂O₅. Finally, in comparative trials precipitation with 2 vol of 2-propanol resulted in recovery yields quite similar to, though generally less than, those obtained when using ethanol.

4.3.2—

Spectrophotometric Method

This method has been claimed to be sensitive, accurate and independent of alginate composition (14). In fact, this statement was not only confirmed for the alginate extracted from *L. hyperborea* (BDH), as shown in Fig. 2, but also for the alginates extracted from *Macrocystis pyrifera* at high-, medium-, and low-viscosity (Sigma), once dissolved in distilled water, 50 mM MOPS-Na or 0.05 M NaCl. In fact, the slope (a_p) of the corresponding standard calibration curves was found to range from 2.9 to 3.4 with standard errors of 0.04–0.1. However, the spectrophotometric method occasionally yielded quite different slopes (a_p in the range of 3.2–4.1) when dealing with the bacterial alginates under study, probably in consequence of their diversity in terms of degree of acetylation and average molecular mass.

This method is by far better in accuracy and precision than the gravimetric one, the coefficient of variation in replicate measurements and the difference between actual and measured alginate concentrations being usually lower than 1 and 5%, respectively. Figure 6 plots the spectrophotometric (P_s) and gravimetric (P_g) determinations of the bacterial alginate present in the supernatants of several shaken-flask and laboratory-fermenter experiments and shows a fairly good correlation coefficient of 0.82. However, it can be noted that the gravimetric measurement overestimates the alginate content assessed by means of the spectrophotometric method as follows:

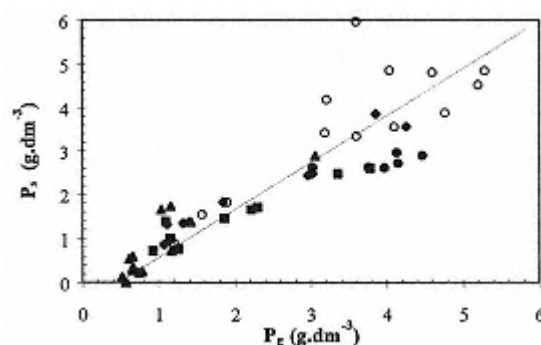


Fig. 6.

Comparison between the spectrophotometric (P_s) and gravimetric (P_g) determinations of the bacterial alginate present in the supernatants of several shaken-flask (open symbols) and laboratory-fermenter (closed symbols) experiments. The broken line was calculated by using the least squares regression (11).

$$P_g = a P_s + b \quad (11)$$

where $a (=0.93 \pm 0.06)$ and $b (=0.47 \pm 0.16)$ are empirical coefficients estimated by the least squares method.

4.4—

Intrinsic Viscosity Estimation

There are several ways of determining the intrinsic viscosity $[\mu]$ from dilute solution viscosity data. In alternative to the exponential regression (Eq. 9) used in Subheading 3.6., the two equations commonly employed for determining $[\mu]$ of food gums are (17):

$$\text{Huggins equation : } \mu_{\text{red}} = [\mu] + k_1 [\mu]^2 c \quad (12)$$

$$\text{Kraemer equation : } [\ln(\mu/\mu_s)]/c = [\mu] + k_2 [\mu]^2 c \quad (13)$$

the equivalence of which involves the following constraint between the Huggins (k_1) and Kraemer (k_2) constants:

$$k_1 - k_2 = 1/2 \quad (13)$$

The extrapolations are usually done for relative viscosity values (μ/μ_s) between 1.2 and 2.0 (28). The Huggins constant (k_1) is considered as an index of the polymer-polymer interaction and generally ranges from 0.3 (for good solvents) to 1.0, its higher values being attributed to some association among the macromolecules (28).

Since most viscosity measurements are performed with capillary viscometers, it should be verified that the shear rate at the capillary wall ($\dot{\gamma}_{\text{max}} = [r \rho g h_{\text{max}}]/[2 L \mu]$, where r is the radius of the capillary, ρ the density of the solu-

tion, g the acceleration of gravity, $h_{\max}$ the maximum head of liquid, L the capillary length, and μ the solution viscosity) be in the low shear-rate Newtonian region of the flow curve (17). When the molecular shape is not spherical or the polysaccharide examined exhibits a very high intrinsic viscosity value (17), the hydrodynamic resistance of the polymer in dilute solutions becomes a function of the orientation the chains assume in the flow field. This makes $[\mu]$ a shear-dependent function and it is therefore necessary to use a low-shear rotational viscometer to obtain the true zero shear-rate viscosity (μ_0) for a series of very dilute alginate solutions. By extrapolating the reduced zero shear-rate viscosity $[(\mu_0 - \mu_s)/(\mu_s c)]$ for c tending to zero, the zero shear-rate intrinsic viscosity $[\mu]_0$ can be determined and used to estimate the average molecular mass of the polymer under study.

Despite the more precise method to determine the intrinsic viscosity value is based upon a combined use of Eqs. 12 and 13 (29), the method based on Eq. 9 in conjunction with Eq. 10 allowed the bacterial alginate M_M to be estimated with an accuracy of the order of $\pm 10\%$, which was regarded as appropriate for evaluating the thickening capability of the bacterial alginates under study.

Acknowledgments

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4—

Production of Schizophyllan

Udo Rau

1—

Introduction.

The filamentous growing fungus *Schizophyllum commune* ATCC 38548 **(1)** secretes a neutral homoglucan (trivial name Schizophyllan) that consists of a backbone chain of 1,3- β -D-glucopyranose units linked with single 1,6-bonded β -D-glucopyranoses at about every third glucose molecule in the basic chain **(Fig. 1)**. The molecular weight varies between 6 and $12 \cdot 10^6$ g/mol **(2)**.

Schizophyllan dissolves in water as a triple helix with protruding pendant β -1,6-linked D-glucose units originating from the outside of the triplex. In DMSO, at temperatures $>135^\circ\text{C}$ and at a pH >12.0 , the triple helix melts to single, randomly coiled chains, equivalent to the reduction of the average molecular weight by one-third **(3)**. Aqueous solutions show thixotropic, pseudoplastic, and viscoelastic behavior. Native suspensions, additionally containing the producing fungus, reveal enhanced non-Newtonian characteristics owing to the filamentous network of the internal woven hyphae.

Many other fungi, like *Sclerotium rolfsii* **(4)**, *Sclerotium glucanicum* **(5)**, *Monilinia fructigena* **(6)**, *Botrytis cinerea* **(7)**, and so forth, are able to secrete the same glucan with a uniform, primary molecular structure. However, these polysaccharides differ substantially in molecular weights and in their tendency to form microgels. These characteristics directly influence their filtration and adsorption behavior when being used as additives for polymer flooding in the scope of enhanced oil recovery. Recent studies have shown that Schizophyllan is very useful for this kind of application **(8)**.

Schizophyllan can also be used to form films almost impermeable to oxygen **(9)**, e.g., for the protection of foods. A further application is the stimulation of the immune system by degraded glucans **(10)**, and especially in Japan, these antitumor glucans are currently used as cancer immunotherapeutic drugs in combination with other chemotherapeutic compounds **(11)**.

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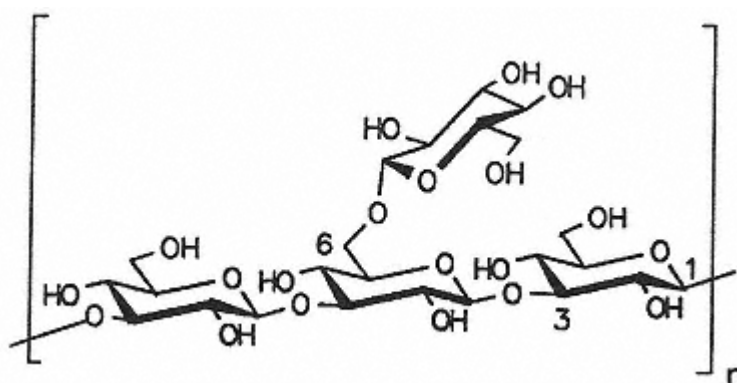


Fig. 1.
Primary molecular structure of Schizophyllan.

The production of Schizophyllan is strongly coupled with growth, and secretion ceased under nitrogen starvation. The gum is as a mucilage either loosely associated with the outer cell wall or released into the medium. Shear stress, created by the agitator used during bioreactor cultivation, reduces pellet growth as well as enhancing release of Schizophyllan from the cell wall. However, too high shear stress causes damage to the hyphae and even the Schizophyllan itself. In addition to this, the resulting cell debris impedes cell separation during subsequent downstream processing. The agitator and speed applied must, therefore, present a compromise between mixing, mass transfer, and Schizophyllan release from the cell wall on the one hand and low shear stress on the fungus and Schizophyllan on the other (12). In addition to the shear stress, an optimum but not maximum oxygen supply is a second key factor for enhanced Schizophyllan production. The subsequent downstream processing of native Schizophyllan suspension is described in detail.

2—

Materials

2.1—

Microorganism

The wood rotting basidiomycete *S. commune* ATCC 38548 is employed to produce the extracellular polysaccharide called Schizophyllan. The fungus is grown on agar slants (7 mL of melted agar in a 20-mL glass tube sealed with a cotton plug) supplemented with 39 g/L potato dextrose agar and 5 g/L yeast extract (both Difco, Detroit, MI). The slants are inoculated with a piece of agar (cut with a small knife) and incubated for 1 wk at room temperature with subsequent storing of the covered slants at 4°C. New slants are inoculated at intervals of 4 wk. For long-time storage, the fungus can also be frozen at -198°C in liquid nitrogen.

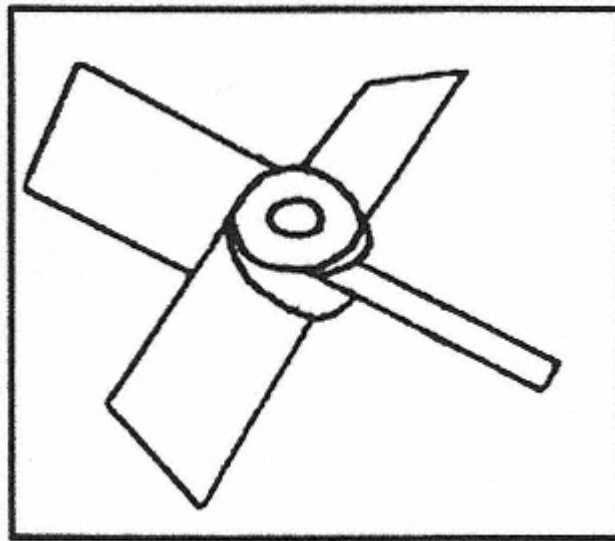


Fig. 2.

2.2—

Medium and Conditions of Cultivation

The medium used for shake flask and bioreactor cultivation (see **Note 1**) consists of (per L deionized water): glucose 30 g, yeast extract 3 g (Ohly, Hamburg, Germany), KH_2PO_4 1 g, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g. Except yeast extract, all chemicals are of analytical grade. The cultivations are performed at 27°C and at an initial pH of 5.3 without pH control.

For the first subculture, a piece of covered agar (see **Note 2**) is used to inoculate 100 mL of medium in 500 mL shake flasks with 2 baffles. After 4 d on a rotary shaker (100 rpm), the second seed with 250 mL medium in 1000-mL shake flasks without baffles is inoculated with 25 mL of homogenized (Ultra Turrax, Ika, Staufen i. Breisgau, Germany) culture suspension (see **Note 3**) and cultivated for 2 d as described before. A longer cultivation time creates a gel-like suspension and, therefore, should be avoided.

Bioreactor cultivations are carried out in a 42-L vessel with a working volume of 30 L (B. Braun Biotech International, Melsungen, Germany). The vessel possesses a height/width ratio of 2 with four baffles mounted inside. Three fourbladed fan impellers (blade pitch 45°, impeller/tank ratio of 0.64; see **Fig. 2**) are installed on the shaft at a distance of 2/3 to the stirrer diameter (0.19 m).

After sterilization of the medium (see **Note 4**), 5% (v/v) of inoculum is added and the culture suspension is gassed with a volume related aeration rate of 0.08 (v/vm, volume of gas/volume of fluid × min).

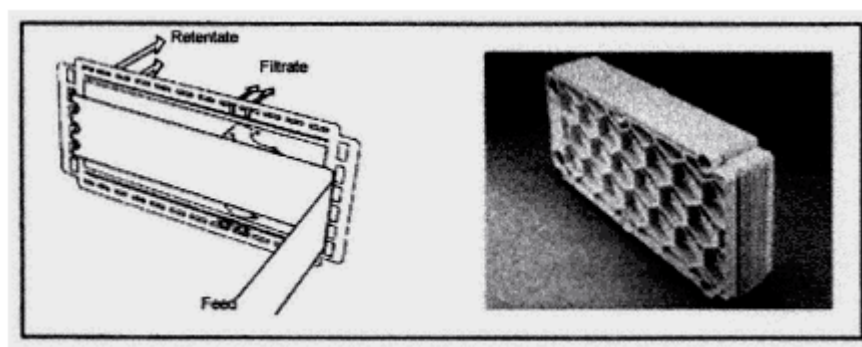


Fig. 3.
Schematic drawing of the PROSTAK™ filtration module (Millipore). Characteristics of the microfiltration membrane:

Active filtration area	0.17 m ² /module
Pore width	0.1 μm
Material of membrane	Polyvinylidenfluorid
pH stability	2–10
Temperature stability	60–80°C, not sterilizable
Max. pressure	4.2 bar
Channel height	1.6 mm
Membranes per module	2
Effective cross section area	4.9 cm ²
Flow distance	33 cm

2.3—

Cell Separation

We use a solid ejecting disk separator (CSA 1, Westfalia, Oelde, Germany) for cell separation with an area equivalent of 1800 m², a solid volume of 0.25 L, and a constant rotation speed of 10,000 rpm equivalent to 8800g at the biggest diameter and 5812g at the smallest diameter of the disk.

2.4—

Microfiltration

Purification and concentration of the cell-free Schizophyllan solution is carried out in crossflow mode by using the PROSTAK™ System (Millipore, Eschborn, Germany), which includes flat channel microfiltration membranes (**Fig. 3**). Recirculation of the fluid is performed by a low shear circuit piston pump (Model 30, Waukesha, WI). The membranes are cleaned with 0.01 *N* NaOH.

2.5—

Viscosity

Shear viscosities of aqueous Schizophyllan solutions are measured by a rotary viscometer (Haake, Karlsruhe, Germany) at different constant shear rates until a constant shear stress results. All measurements are carried out at 25°C.

3—

Methods

This section is divided into four parts. First, the analytical procedure for the determination of the culture parameters is explained. After a detailed description of a bioreactor cultivation using *S. commune* with subsequent downstream processing of the Schizophyllan produced follows a rheological characterization of the purified Schizophyllan solution.

3.1—

Analysis

3.1.1—

Cell Dry Weight.

For the determination of the cell dry weight, the culture suspension has to be diluted appropriately (see Note ⁵) with deionized water, subsequent homogenized (60 s in a 250 mL vessel, Waring Blender, Bender und Hobein, Zürich, Switzerland) in order to separate the cell-associated Schizophyllan from the mycelium and centrifuged at 15,000*g* for 20 min. Wash the pellet twice with deionized water, and dry it to constant weight (48 h) at approx 50 mbar and 40° C.

3.1.2—

Schizophyllan

Add 2 vol of propan-2-ol to the cell-free supernatant that is subsequently stored at 4°C for 1 h to complete precipitation. After centrifugation and washing with 70% (v/v) propan-2-ol the Schizophyllan is dried to constant weight as described in **Subheading 3.1.1**.

3.1.3—

Glucose

For this quick and inexpensive procedure a commercial blood-sugar analyzer is used. Therefore, the cell-free supernatant (4 vol) has to be neutralized to pH = 7.0 with 1 vol 1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$. If the glucose content is still too high, dilute the solution with deionized water. The applied accutrend α -D-glucose analyzer (Boehringer Mannheim, Germany) needs a sample volume of 20 μL . This determination is performed at room temperature.

3.1.4—

Viscosity

The shear viscosities of aqueous cell-free Schizophyllan solutions are measured by a rotational viscometer (Rotovisco RV 100, Haake, Karlsruhe, Germany) at 25°C. A constant shear stress at constant shear rate indicates the end of measurement. This is a fast approach to the quality of the purified Schizophyllan. In order to compare different Schizophyllan solutions, the measurement has to be carried out at constant concentration and shear rate.

3.2—

Bioreactor Cultivation

Fill the 30-L bioreactor mentioned in **Subheading 2.2**. with 28.5 L of water and dissolve all media compounds under stirring. The sterilization procedure is

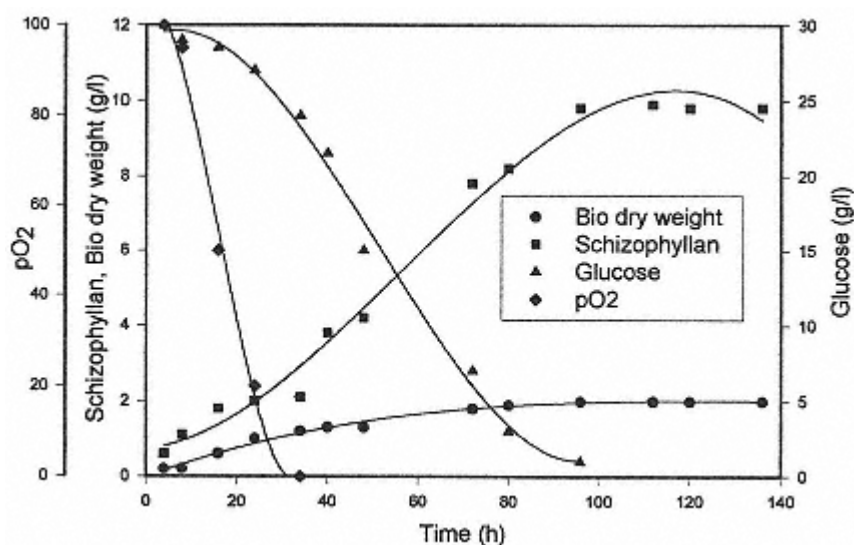


Fig. 4.

30-L Batch cultivation of *S. commune* equipped with three-fan impellers at 100 rpm, 27°C, an initial pH of 5.3 and an aeration rate of 0.083 (v/vm).

carried out at 121°C and 1 bar for 20 min. The common sterilization of glucose and yeast extract is possible in this composition (**Subheading 2.2.**). Unconsumable compounds essential for *S. commune* are not formed. Initiate the cultivation by adding 1.5 L of the second seed (see **Subheading 2.2.**) as inoculum. After approx 4 d, the glucose is consumed by *S. commune*, production of Schizophyllan ceases and the cultivation is terminated (see Note 6). A typical course of cultivation (13) is shown in **Fig. 4**.

The formation of extracellular Schizophyllan is strongly associated with growth. Therefore, if *S. commune* is not able to grow, then the formation of Schizophyllan ceases also. In other words, the production of Schizophyllan is not feasible using resting cells, for example, under nitrogen limited conditions (14). However, an optimum in oxygen supply and shear stress (see Note 7) are the key factors for enhanced Schizophyllan production with *S. commune*.

As can be seen from **Fig. 4**, most Schizophyllan is produced when the fungus is oxygen-limited, that is, in a time range between 30 and 100 h. During this time interval, the partial pressure of oxygen in the liquid phase is almost zero and the additional formation of ethanol occurred (not shown in **Fig. 4**). This characteristic indicates that the fungus needs an optimum oxygen supply for a maximum of Schizophyllan secretion. This fact is proved without doubt by the determination of an optimum constant specific oxygen uptake rate (oxygen consumed per time [g/L h] divided by the actual biomass [g/L]) from con-

tinuous cultivations in an oxygen-limited chemostat (15). Related to primary mycelial (homogeneous) growth of the fungus and using exactly the same bioreactor setup as well as cultivation conditions mentioned above, then the optimum specific oxygen uptake rate is equal to 0.04 h^{-1} (16). However, if another configuration is used, this specific value can change drastically and has to be determined individually by an in-house cultivation combined with exhaust analysis. The control of a constant specific oxygen uptake rate during a batch cultivation by, for example, the impeller speed enables an 20% increase of the Schizophyllan yield from 10 g/L (Fig. 4) to 12 g/L (16). One has individually to decide whether the relatively high expense of analysis and control is justified by this increased Schizophyllan yield.

3.3—

Downstream Processing

3.3.1—

Cell Separation

Dilute the harvested native culture suspension with deionized water to $\leq 1 \text{ g/L}$ Schizophyllan. Homogenize (see Note 8) and stabilize the suspension against infections with 5 g/L formic acid or 0.5 g/L formaldehyde (see Note 9). A crude separation of the cells (95%) is achieved by continuous centrifugation with a solid ejecting disk separator (see Subheading 2.3.). Best results in clarification (see Note 10) are yielded when the 1:10 diluted and homogenized suspension (1 g/L Schizophyllan, 0.2 g/L biomass, see Fig. 4) is fed at 10 L/h with solid discharge at a time interval of 15 min. The resulting supernatant contains only small amounts of hyphal fragments (concentration $<0.1 \text{ g/L}$), which can easily be separated by dead-end filtration using glass-fiber filters (13400 K293, Sartorius, Göttingen, GER). The cell-free diluted Schizophyllan solution has to be purified and concentrated in a next step.

3.3.2—

Crossflow Microfiltration

Owing to parallel investigations using different crossflow systems (17), it is strongly recommended that the application of PROSTAK™ flat membrane ($0.1 \mu\text{m}$) modules (see Subheading 2.4.) operating in crossflow mode for the purification (diafiltration mode) and concentration (Fig. 5) of the cell-free Schizophyllan solution.

Connect three filtration modules in series each with a area of 0.17 m^2 . The tangential feed velocity of the retentate varies between 1 and 6.6 m/s. Best results related to a high permeation rate are achieved when the tangential feed velocity is at its individual maximum avoiding a transmembrane pressure $>0.8 \text{ bar}$ (see Note 11). This optimum adjustment can be easily attained in the purification step using the diafiltration mode (Fig. 5). In this context, purification means that the Schizophyllan molecules are fully rejected and low molecular compounds ($<0.1 \mu\text{m}$), such as proteins, glucose, and salts, permeate

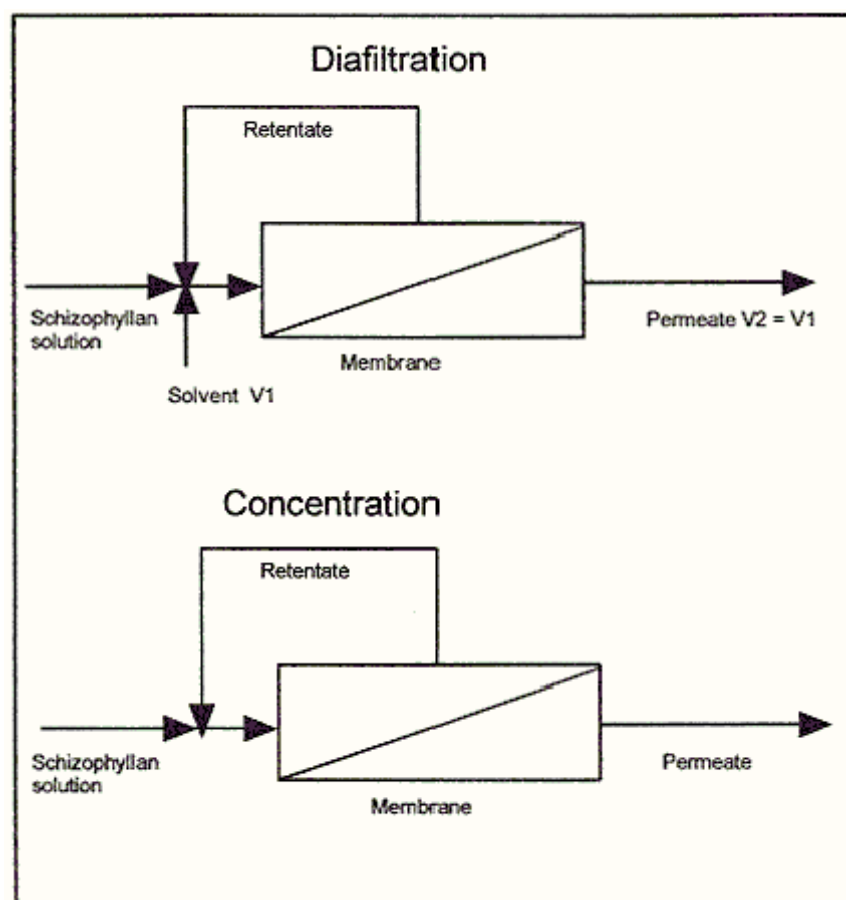


Fig. 5.
Schematic setup of the crossflow microfiltration for the purification (diafiltration) and concentration of cell-free Schizophyllan solutions (V = volume feed; L/h).

through the membrane. Let the output permeate flow equal the input solvent (water) flow, so that the volume of the retentate remains constant. This procedure enables only a small decrease of the permeation rate, leveling off at approx $30 \text{ L/h} \cdot \text{m}^2$. The grade of purification depends on the application of the Schizophyllan and has to be determined individually (e.g., protein content).

Stop the input of water flow after sufficient purification of the Schizophyllan solution and concentrate the solution subsequently to a final content of 10 g/L at maximum (see Note [12](#)). During this process, the negative influence of fouling at the membrane surface is increased with the consequence of a continual decrease in the permeation rate (**Fig. 6**). A highly viscous, colorless,

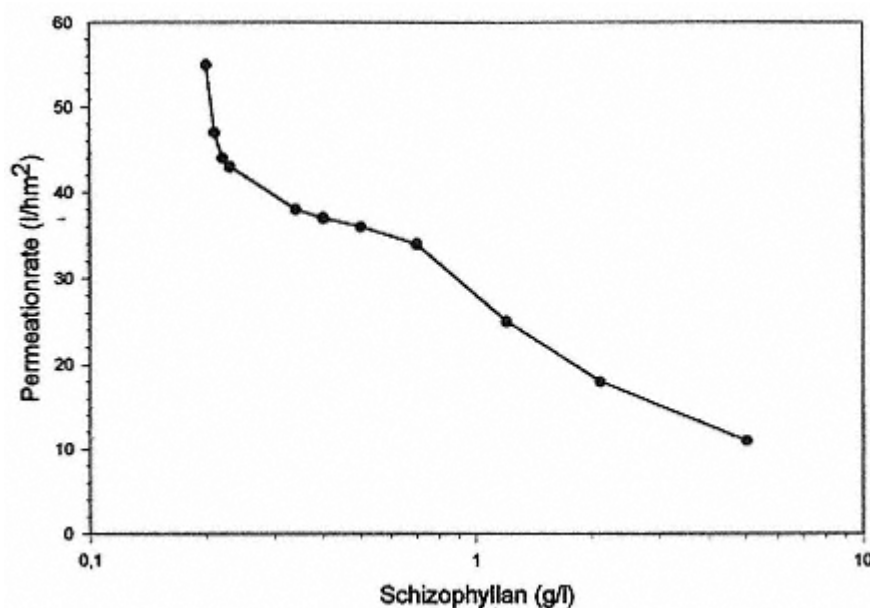


Fig. 6.
Decrease of the permeation rate during the concentration run of a cell-free Schizophyllan solution applying crossflow microfiltration.

and transparent solution is the result of this procedure. Drying or lyophilization of the product solution has to be avoided, because only 50% (w/w) of the dried Schizophyllan is resolvable in water. Otherwise dimethylsulfoxide dissolves the dried Schizophyllan to 100%. However, this solvent degrades the triple helix to single-coiled chains with drastic reduction of viscosity.

3.3.3—

Characterization of the Schizophyllan Solution

Aqueous Schizophyllan solutions show pseudoplastic flow behavior. Thus, the viscosity is decreased with increasing shear rate (**Fig. 7**). Owing to this flow behavior, an individual viscosity is strongly connected to a single shear rate and characterizes the quality of the Schizophyllan, because in most applications, a high viscosity at low Schizophyllan concentration is required. Furthermore, the shear viscosity depends on the concentration of the Schizophyllan. For example, **Fig. 7** shows the flow behavior of a solution with 5 g/L Schizophyllan. We choose the lowest shear rate (0.3 s^{-1}) of our viscometer (see **Subheading 2.5.**) for the comparison of different concentrated solutions yielding viscosities $>10 \text{ Pa s}$ for concentrations $>5 \text{ g/L}$. The mean value of a 0.3 g/L solution (shear rate 0.3 s^{-1}) varies between 50 and 150 mPa s depending on the quality of the Schizophyllan.

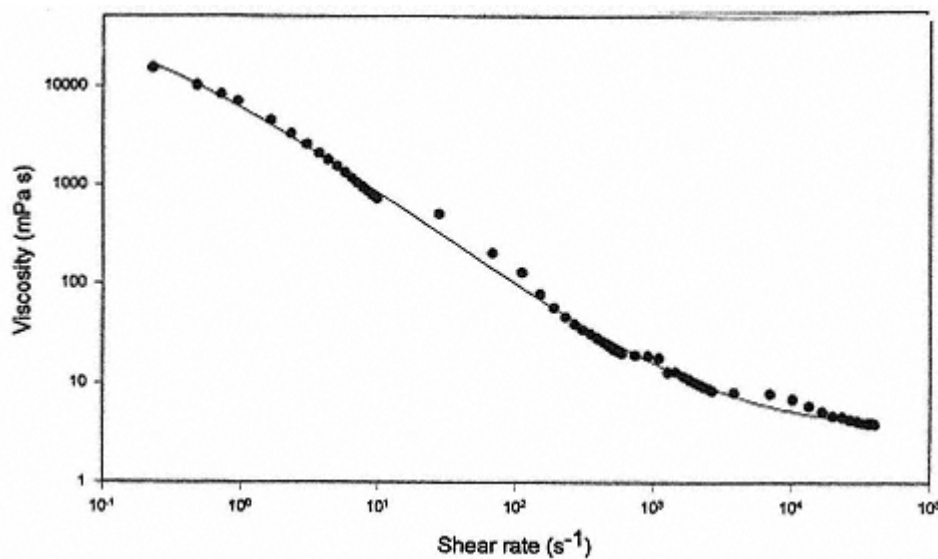


Fig. 7.
Pseudoplastic flow behavior of an aqueous Schizophyllan solution (5 g/L). For conditions of measurement, see **Subheading 2.5**.

4—

Notes.

1. A light-yellow-colored Schizophyllan solution is created by the addition of yeast extract to the medium. If a colorless product solution is needed, use the following medium (per L deionized water): 33 g glucose, 13 g sorbitol, 1 g KH_2PO_4 , 3.8 g $MgSO_4 \cdot 7H_2O$. The compounds 0.6 g urea, 0.64 g thiamine·HCl and 1 mL of a trace element solution should be added as sterile-filtered (0.2- μm) solution. The trace element solution contains (per L deionized water): 17.5 g Na_2EDTA (Titriplex III) (dissolve first), 2.8 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 2.4 g $FeSO_4 \cdot 7H_2O$, 0.08 g $CoSO_4 \cdot 7H_2O$, 0.2 g $CuCl_2 \cdot 2H_2O$, 14 g $NaBO_2 \cdot 4H_2O$, 0.08 g $Na_2MoO_4 \cdot 2H_2O$, 0.24 g $NiCl_2 \cdot 2H_2O$. All chemicals were of analytical grade.
2. As an alternative to agar slants, homogenized submerged cultures from shake flasks stored at 4°C, but not longer than 2 wk, can also be used as inoculum.
3. Pour 100 mL of the first subculture into a 250-mL presterilized beaker (sealed with aluminum foil). Also immerse a presterilized mixer rod into the suspension, and homogenize for 30 s.
4. Use a homogeneous culture suspension. Otherwise, if pellets with a diameter >2 mm are recognizable, apply the homogenization procedure (see Note [3](#)) again. The inoculum (1.5 L, second seed) is poured into a 3-L glass vessel equipped with two pipes. One is connected at the top of the vessel and sealed by a sterile membrane filter (0.2 μm). The other pipe at the bottom is attached to a silicone tube and also sealed by an inoculation kit (B. Braun Biotech International, Melsungen, GER). If the suspension is too viscous and does not flow into the

bioreactor, mount the pressure tube of the pH-electrode (0.5 bar) at the end of the sterile filter.

5. Dilute the native culture suspension with water if the concentration of dry biomass and/or Schizophyllan exceeds 2 g/L. The resulting concentration should not exceed 1 g/L. Work accurately at this dilution step, and weigh the culture suspension instead of measuring the volume. Use a pipet with a wide-open outlet. For example, dilute 20 g of suspension, density approx 1050 kg/m³, with 80 mL of water. Do not use the sucked volume to calculate the dilution, because the high viscosity prevents the complete outflow of the suspension.

6. Prolonged cultivation under carbon-limited conditions leads to the release of Schizophyllan-degrading enzymes (β -glucanases). These enzymes cause a slight increase in glucose concentration accompanied by a decrease in concentration of the Schizophyllan and a sharp drop in the shear viscosity of the cell-free Schizophyllan solution. The reason for this behavior is that small fragments of the Schizophyllan serve as carbon source for the fungus.

7. For optimum results, use a four-bladed fan impeller, because mixing of this highly viscous suspension requires a proper agitator that allows short mixing times, high mass transfer, and Schizophyllan release from the cell wall on the one hand, and low shear stress on the fungus and Schizophyllan on the other. The meaning of optimum results is a combination of a high yield of Schizophyllan and a low ratio of biomass/Schizophyllan. Other impellers, such as the conventional Rushton turbine or Intermig (Ekato, Schopfheim, GER), are also applicable for cultivation of *S. commune*. However, you achieve poorer results **(12)**.

8. An intensive homogenization is necessary for the release of cell-wall-associated Schizophyllan to decrease the loss during downstream processing. Large-scale homogenization can be carried out by recycling the culture suspension with the aid of a gear pump, applying jet or nozzle systems or with high-speed agitation. The efficiency of the homogenization procedure applied is proportional to the Schizophyllan content of the resulting supernatant.

9. Do not use sodium azide as stabilizer, because it only works well against bacteria. Furthermore, the stabilizing effect disappears in a few days owing to self-decomposition in aqueous solution with release of nitrogen.

10. We tested different centrifugal separators operating in continuous or batch mode also with variable rotation speed. However, if larger volumes have to be separated by centrifugation, small amounts of hyphal fragments are observed always in the supernatant. Crossflow microfiltration also failed as the sole procedure for cell separation owing to instantaneous clogging of the membrane or ineffective separation characteristics **(17)**.

11. It is very important for an effective filtration that the transmembrane pressure:

$$\Delta P_{TM} = (P_{in} + P_{out}/2) - P_{permeate} \quad (1)$$

does not exceed 0.8 bar, because higher transmembrane pressures do not contribute to higher permeation rates. On the contrary, in this highly viscous pseudoplastic fluid system, a $\Delta P_{TM} > 0.8$ bar is combined with increased transport of

dissolved Schizophyllan molecules toward the membrane surface. The result is an enhanced fouling with extraordinary increase of the gel-layer thickness. The only way to achieve a higher constant permeation rate is the increase of the tangential feed velocity. However, be careful with this procedure, because owing to higher drag reduction, the transmembrane pressure simultaneously increases.

12. Using crossflow microfiltration, a concentration of Schizophyllan higher than 10 g/L is not recommended owing to gel-like behavior of these solutions. A yield stress is formed, and thus, the flow stops in regions with low shear stress. If higher concentrations are needed, only a rotating evaporator is applicable (≤ 20 g/L).

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5—

Enzymatic Synthesis of Cellulose

Shiro Kobayashi, Shin-ichiro Shoda, Michael J. Donnelly, and Stephen P. Church

1—

Introduction

Cellulose, a homopolysaccharide whose glucose units are connected through $\beta(1\rightarrow4)$ glycosidic linkages, has been of great interest in many scientific and application fields as polymeric drugs and new biomaterials **(1)**. So far, highly pure samples of cellulose and its derivatives have been produced for functional macromolecules, specimen for basic crystallographical research, enzyme substrates, and so on starting from various kinds of naturally occurring celluloses. The elongation of cellulose chain in vivo involves the addition of a nucleotide monomer, uridine diphosphate-glucose (UDP-glucose), to the nonreducing end of cellulose or cello-oligomers. The transformation of the naturally occurring cellulose into pure cellulose or cello-oligomers involves complicated processes such as the removal of hemicellulose and lignin, acetylation of hydroxyl groups, acidic cleavage of the cellulose backbone, and fractionation by chromatography. Therefore, the development of a new methodology for a convenient laboratory-scale preparation of cellulose and cello-oligomers has been strongly demanded.

In spite of many attempts of cellulose to synthesize in vitro, the construction of the cellulose backbone had never been realized for more than half a century **(2–4)**. Although chemical synthesis of cellulose via the cationic ring-opening polymerization of an orthoester derivative of glucose was recently reported **(5)**, the method requires multi-step processes, including the regioselective blocking and deblocking of a hydroxy group in the glucose moiety at the stage of monomer synthesis. Furthermore, the protecting group for the hydroxyl groups must be removed at the final stage of synthesis.

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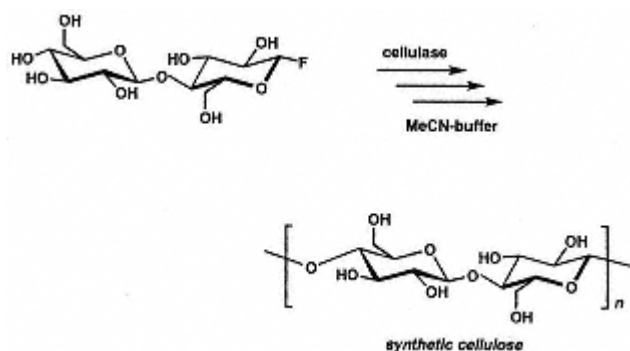


Fig. 1.

The enzymatic polymerization of β -cellobiosyl fluoride monomer catalyzed by cellulase in acetonitrile buffer.

Enzymes over the last decade have been used in a remarkably wide range of organic synthetic reactions of both academic and industrial interest (6,7). However, despite the fact that a vast array of polymers are synthesized and modified *in vivo* by enzymatic action, it is only in the last several years that attention has been given to the usefulness of this area to polymer science (8,8a-c). The use of a glycosyl hydrolase as catalyst for selective synthesis of complex polysaccharides has become a vivid topic in polymer synthesis (9-13). One example is the first successful *in vitro* synthesis of cellulose via a nonbiosynthetic pathway by utilizing cellulase as catalyst, where the enzymatic synthesis of cellulose can be achieved via polycondensation of β -cellobiosyl fluoride monomer (9,10) (Fig. 1). The present chapter provides precise information on the production of synthetic cellulose and cello-oligomers catalyzed by cellulase on a laboratory scale.

The synthetic route for the preparation of β -cellobiosyl fluoride monomer is shown in Fig. 2. The acetoxy group at the anomeric position of commercially available cellobiose octa-acetate is converted to the fluorine atom by using the following procedures: (1) The octa-acetate is brominated by treating it with hydrogen bromide in acetic acid. (2) The introduction of the fluorine atom at the C-1 position is achieved by treating the bromide with silver fluoride in acetonitrile. (3) Finally, all acetyl groups of the resulting fluoride are deprotected by an alkaline methanolysis.

The purity of β -cellobiosyl fluoride monomer is very important for the cellulase-catalyzed polymerization; at least 90% purity is required to make the polymerization proceed effectively. The purity of the monomer strongly depends on the final step of the monomer synthesis of alkaline methanolysis of the acetyl groups. The reaction time for the methanolysis as well as the successive neutralization by an ion-exchange resin should be as short as possible to

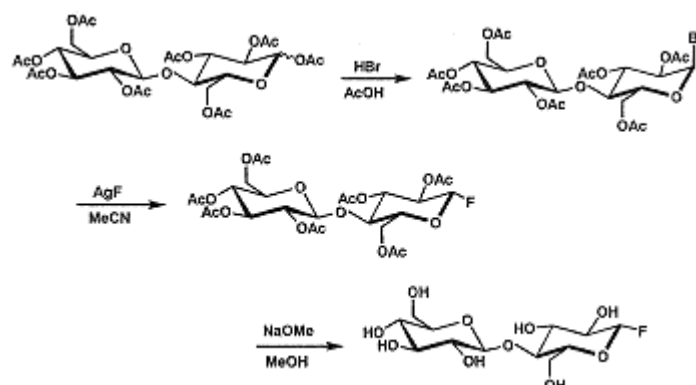


Fig. 2.
Synthetic route of β -cellobiosyl fluoride monomer starting from cellobiose octa-acetate.

avoid the nucleophilic attack of methanol at the anomeric center and acidic hydrolysis of C-F bond, respectively.

The experimental procedure for the enzymatic polymerization is quite simple. In a test tube, a solution of cellulase from *Trichoderma viride* is added to a solution of β -cellobiosyl fluoride monomer in a mixed solvent of acetonitrile/acetate buffer. The addition of acetonitrile is essential for the cellulase catalyst to promote the reverse reaction of the hydrolytic decomposition. The polymerization perfectly controls the regio- and stereochemistry of the reaction, giving rise to the synthetic cellulose (degree of polymerization >22). Alternatively, under the reaction conditions of a higher substrate concentration or higher acetonitrile concentration, water-soluble cello-oligomers (degree of polymerization <8) are produced predominantly. In the present case, conditions are selected to produce predominantly polymeric material. After inactivating the enzyme, the reaction mixture is purified, yielding a water-insoluble white powdery material: synthetic cellulose and a mixture of cello-oligomers. **Figure 3** shows the flow chart for the preparation of synthetic cellulose (the water-insoluble part) as well as cello-oligomers (the water-soluble part) by the present method.

Structural studies on the water-insoluble component of the reaction product (synthetic cellulose) have been previously carried out using X-ray diffraction (powdered sample), infrared (IR) spectroscopy (KBr disc) and solid state ^{13}C NMR spectroscopy (**9**). In the first case, the diffractograms showed differences between the synthetic cellulose, mercerized cellulose (representative of cellulose crystal structure type II), and a sample of natural cellulose (wood pulp of DP $\sim$ 200, cellulose crystal structure type I). The spectroscopic techniques showed small differences between the spectra of the different types of

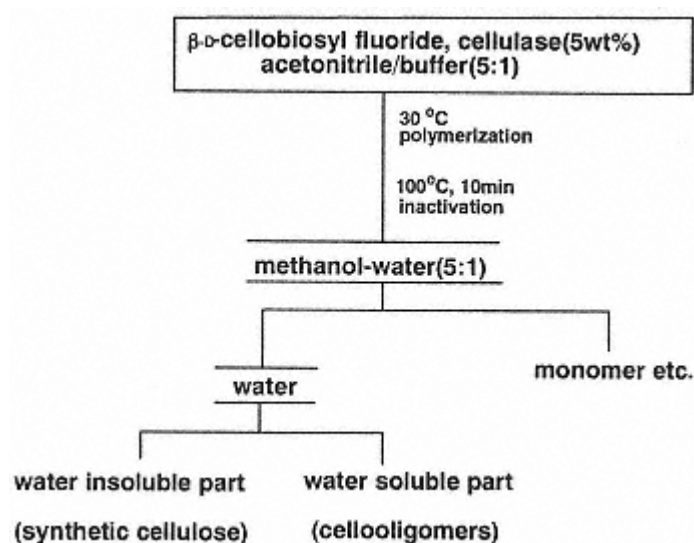


Fig. 3.

Flowchart for the preparation of synthetic cellulose (the water-insoluble part) and cello-oligomers (the water-soluble part).

celluloses, all of which were consistent with the synthetic cellulose existing in the cellulose type II form and possessing high crystallinity. However, some of these measurements involved treatment of the sample before analysis, and it is known that cellulose allomorphs may be reversibly transformed into each other.

In order to further characterize the nature of the synthetic cellulose, Fourier transform (FT)-IR and FT-Raman spectra have now been measured for the native sample (14). The spectroscopic analysis was deliberately carried out without sample preparation, and hence avoided any possible modification of the sample. Reference material samples of regenerated cellulose (Viscose, representative of cellulose II) and pulp (cellulose I) were also analyzed under the same conditions and the results compared and contrasted in order to confirm which allomorph is formed.

2—

Materials

2.1—

Reagents and Solvents for Preparation of β-Cellobiosyl Fluoride

1. Cellobiose octa-acetate is completely dried *in vacuo* before use.
2. Acetic acid solution of hydrogen bromide (33%) is stored at 4°C.
3. Silver fluoride is completely dried *in vacuo* at 100°C for several hours before use.
4. Acetonitrile is purified by distillation over calcium hydride before use (see Note 1).
5. Methanol is distilled from magnesium before use (see Note 1).

2.2—

Buffer and Enzyme for Polymerization of β -Cellobiosyl Fluoride

1. Acetate buffer (pH 5.0) is prepared by mixing an acetic acid (0.05 M) solution and a sodium acetate (0.05 M) solution with monitoring the pH value with a pH meter.
2. Cellulase from *Trichoderma viride* (Onozuka R-10) is purchased from Yakult Co. (Tokyo). This commercially available enzyme can be utilized for enzymatic polymerization without purification. The powder should be stored in a screw-cap bottle protected by containment in a plastic bag. (see Note [2](#)).

3—

Methods

3.1—

Procedure for Monomer Synthesis

1. Added to a solution of 25.0 g cellobiose octa-acetate in 150 mL chloroform is a mixture of hydrogen bromide (33% solution in acetic acid) (42 mL) and 10 mL chloroform dropwise, using a dropping funnel at 0°C and the resulting reaction mixture is stirred for 4 h at the same temperature.
2. The resulting solution is washed with ice water using a separatory funnel until the pH of the water phase becomes almost neutral. The organic layer is collected and dried over anhydrous sodium sulfate.
3. After filtration, the organic solvent is removed under a reduced pressure below 35°C and the residue is washed with petroleum ether or *n*-hexane to crystallize the product. The resulting crystals are filtered and dried *in vacuo* to give 24.3 g α -cellobiosyl bromide hepta-acetate (see Note [3](#)).
4. In a two inlet flask shaded by aluminum film, 7.0 g silver fluoride is vacuum pumped for 1 h on a 100°C oil bath. After being cooled to room temperature, the silver fluoride is suspended in 50 mL acetonitrile.
5. To this suspension a solution of 12.1 g α -cellobiosyl bromide hepta-acetate in 100 mL acetonitrile is added at room temperature. The reaction mixture is stirred vigorously at room temperature until the complete consumption of α -cellobiosyl bromide hepta-acetate is confirmed by a thin layer chromatography (silica gel plates, ethyl acetate/*n*-hexane 2:1 [v/v]) (see Note [4](#)).
6. Silver salts were filtered off by using G4 glass filter with celite and the filtrate is concentrated to dryness under a reduced pressure. The crude product is further purified by column chromatography (silica gel 60, 70–230 mesh) with ethyl acetate/*n*-hexane (2/3[v/v]) as eluent.
7. Added to a solution of 3.1 g β -cellobiosyl fluoride hepta-acetate in 100 mL methanol is a freshly prepared solution of 2.5 mL, 0.3 M sodium methoxide in methanol at 0°C (ice-water bath). The deacetylation reaction is monitored by thin-layer chromatography (silica gel plates, chloroform/methanol 2/1[v/v] as eluant) (see Note [5](#)).

8. The reaction mixture is treated with excess of Amberlite IR-120 (H^+) until the solution becomes almost neutral (*see* Note [5](#)). The resin is filtered and the filtrate is evaporated to dryness under reduced pressure. The residue is desiccated *in vacuo* to give 1.7 g β -cellobiosyl fluoride (*see* Notes [6](#) and [7](#)).

3.2—

Procedure for Enzymatic Polymerization.

1. The substrate monomer of β -cellobiosyl fluoride (334 mg, 0.97 mmol) was dissolved in 5.6 mL 0.05 M acetate buffer, pH 5.0.
2. Acetonitrile (33 mL) was added to the above solution (see Note [8](#)).
3. Added to this mixture is a 1.0-mL 0.05 M acetate buffer solution of crude cellulase (17 mg, 26 U, 5.1 wt % for the substrate) from *Trichoderma viride*, and the resulting mixture was kept at 30°C for 12 h (see Note [9](#)).
4. The resulting suspension was heated at 100°C for 10 min to inactivate the enzyme and was poured into an excess amount of methanol/water 5/1(v/v) (see Notes [10](#) and [11](#)).
5. The insoluble part was collected by filtration. Further purification was achieved by suspending the product in water and by successive filtration, giving rise to the water-insoluble part of the white powdery materials: synthetic cellulose (180 mg) (see Note [12](#)).
6. On the other hand, the filtrate was concentrated *in vacuo* to give a white powdery water-soluble part: cello-oligomers (32 mg) (see Note [13](#)).

3.3—

Procedure for Spectroscopic Analysis

3.3.1—

FT-Infrared Spectroscopy

The sample is placed on a rock salt plate and presented for viewing under the IR microscope (Spectra-Tech IRPLAN microscope/Nicolet 740 FTIR spectrometer). Suitable sample areas are located and masked down using the aperturing system. Samples are measured at 4 cm⁻¹ and 2 cm⁻¹ resolution over 1000 co-added scans (see Notes [14](#) and [15](#)).

3.3.2—

FT-Raman Spectroscopy

The sample is placed in the spectrometer's solids holder and analyzed using a Bruker IFS 66/FRA 106 FT Raman Spectrometer. The excitation laser used is an ADLAS Nd: YAG operating at a wavelength of 1064 nm and a power of 300 mW. The spectra are recorded at 4 and 2 cm⁻¹ resolution over ~2000 co-added scans (see Notes [14](#) and [15](#)).

3.3.3—

Allomorph Characterization by FT-IR and FT-Raman Spectroscopy

The FTIR spectrum for the synthetic cellulose, for the region 4000–2200 cm⁻¹ is given in **Fig. 4** and for the region 2000–650 cm⁻¹ in **Fig. 5**. In each case the precise frequency of absorption is also given for each detected peak. The corresponding Raman spectrum for this material is given in **Fig. 6** again with the precise Raman shift frequencies given at the top of the spectrum. These spectra are compared with those obtained for pulp and Viscose in **Fig. 7** (FTIR spectra) and **Fig. 8** (FT-Raman spectra) (see Note [16](#)).

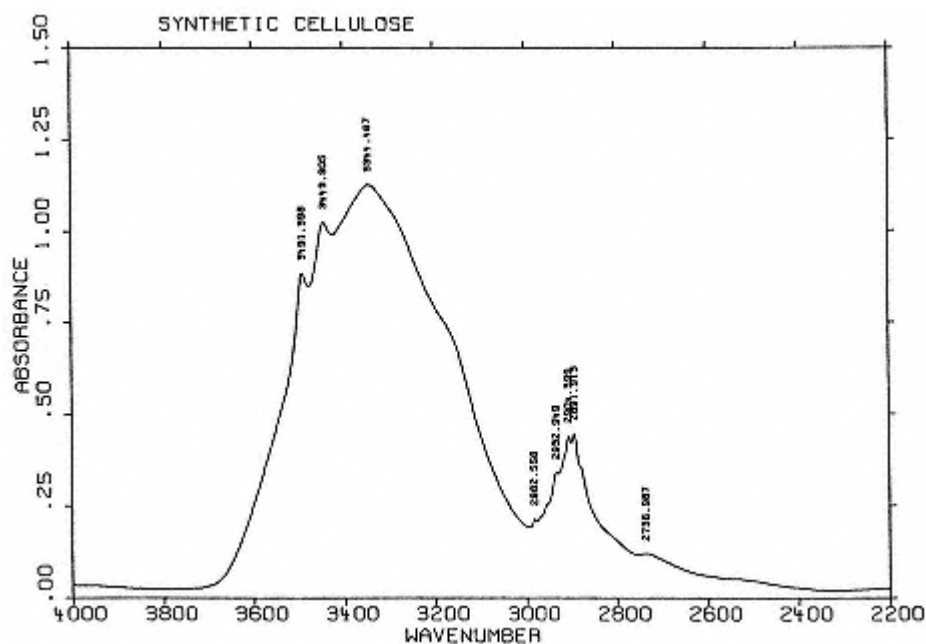


Fig. 4.

FT-IR spectrum of synthetic cellulose (4000–2000 cm^{-1}).

The striking feature of the FTIR spectrum of the synthetic cellulose, unusual for the analysis of these types of polymers, is the wealth of detail obtained. This is indicative of a highly crystalline sample. The most significant feature of **Fig. 4** is the 2 intramolecular hydrogen-bonding bands at 3491 and 3444 cm^{-1} . These two bands are only observed in the spectra of crystalline cellulose type II and are particularly prominent in the spectrum of the synthetic cellulose. They are not observed in the poorly crystalline regenerated cellulose Viscose nor in the spectrum of the pulp sample (crystalline cellulose type I).

Also of interest in **Fig. 4** is the splitting of the C-H stretching manifold ($\sim 2900 \text{ cm}^{-1}$) that has not previously been seen in the spectra of materials of this type (see Note [17](#)). A further point of interest for the synthetic cellulose is the intensity of the water adsorption peak seen at 1643 $^{-1}$ in **Fig. 5**. This peak is stronger than usual, indicative of the sample containing a relatively high level of water compared with the other types of celluloses analyzed.

The Raman spectrum for the synthetic cellulose (**Fig. 6**) also shows great detail and may also be rationalized in terms of a highly crystalline cellulose type II. The most useful diagnostic features occur in the region of 600–500 cm^{-1} where 2 signals of approx equal intensity are observed (576 and 518 cm^{-1}). These peaks are particularly characteristic of cellulose type II. For the regenerated cellulose

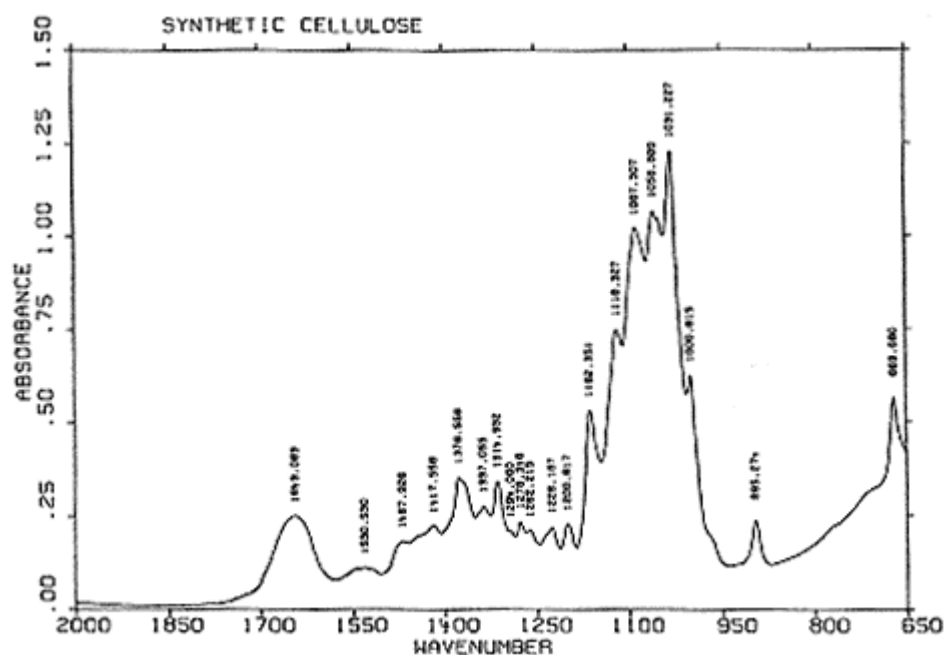


Fig. 5.

FT-IR spectrum of synthetic cellulose (2000–650 cm^{-1}).

(Viscose) the peak at 576 cm^{-1} is of similar intensity to that obtained for the synthetic cellulose, but the peak at 518 cm^{-1} is only very poorly resolved (**Fig. 8**). The reverse trend is seen for the sample of the pulp. The equal intensities of these two peaks is therefore additional proof that the synthetic cellulose is the type II crystalline form. In addition it is noteworthy that there are other uncharacterized differences between the spectra of **Fig. 8** over the region $\sim 500\text{--}300\text{ cm}^{-1}$.

4— Notes

1. The purification of the solvents acetonitrile and methanol is a very important factor which affects the yield of β -cellobiosyl fluoride. Freshly distilled solvents are recommended.
2. The cellulase powder should be stored at low temperature and with prevention of water ingress. This minimized clumping and, hence, inaccuracies when weighing out small amounts of the catalyst. It also lowers the risk of enzyme deactivation caused by hydrolytic or microbial effects. Efforts should be made to minimize the risk of exposure to enzyme powder so as to reduce the risk of an allergic reaction.

The crude enzyme contains several hundred proteins according to SDS/PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis) analysis. If neces

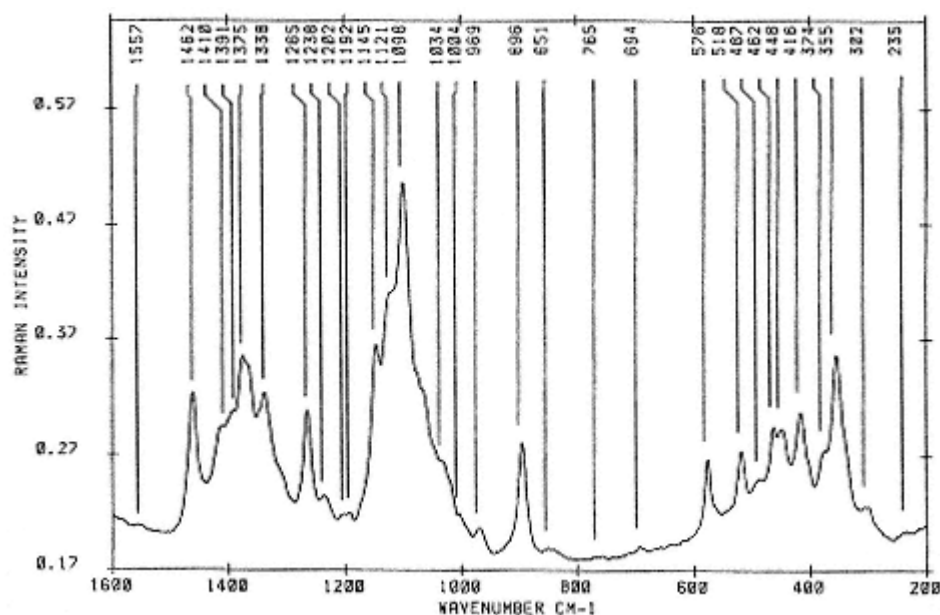


Fig. 6.
FT-Raman spectrum of synthetic cellulose.

sary, the crude cellulase can be purified as follows to give a fraction with a higher catalytic activity for the polymerization of β -cellobiosyl fluoride: Two grams of the crude cellulase is added to 40 mL of 0.01 *M* sodium acetate buffer (pH 5.0) and the insoluble material is removed by centrifugation at 50,000_g for 30 min. Ammonium sulfate fractionation leads to a precipitate (between 37 and 45% saturation) that is pelleted by centrifugation at 50,000_g. The pellet is dissolved in 10 mL of 0.01 *M* sodium acetate buffer, pH 5.0, and loaded into a Bio-Gel P-10 desalting column (30 × 500 mm) with a flow rate of 25 mL/h at 4°C. The void-volume fractions are pooled and concentrated. One milliliter of a Bio-Lyte Ampholyte pH 3–10 (40% w/v), 1 mL of Bio-Lyte Ampholyte pH 3.0–5.0 (20 % w/v), and deionized water are added to make a final volume of 55 mL. This solution is loaded into a Rotofor preparative isoelectric-focusing cell. Fractions showing the greatest cellulose productivity are pooled and concentrated by using a Centricon-30. This concentrated material is applied to a BioGel P-60 column (20 × 900 mm) with a flow rate of 7.5 mL/h at 4°C. Fractions eluted from the P-60 column are analyzed and the most active fraction (in terms of ability to synthesize cellulose) is taken as the P-60 fraction.

3. α -cellobiosyl bromide hepta-acetate is to be stored below 0°C. ^1H NMR spectrum (CDCl_3) δ 6.52 ppm (d, $J = 4.2$ Hz, 1H, anomeric proton of reducing end unit).

4. In order to avoid the decomposition of the starting α -cellobiosyl bromide hepta-acetate and the resulting β -cellobiosyl fluoride hepta-acetate completely dried

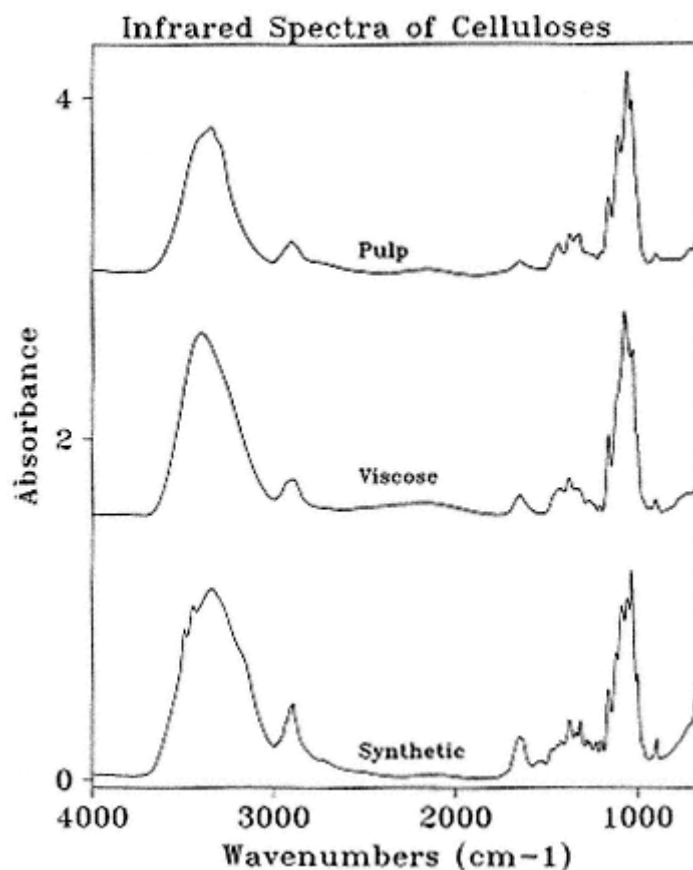


Fig. 7.
FT-IR spectra of pulp, viscose, and synthetic cellulose.

acetonitrile must be used for this substitution reaction. Prolonged reaction should be avoided because the resulting β -cellobiosyl fluoride hepta-acetate may anomerize to the thermodynamically more stable α -isomer.

5. The deprotection of acetyl groups from the resulting β -cellobiosyl fluoride hepta-acetate requires completely dried methanol as solvent. For this deprotecting process, reaction time is very important. The deprotecting process should be monitored as often as possible by a thin-layer chromatography. The reaction must be stopped by addition of ion-exchanging resin as soon as all acetyl groups are removed. Prolonged reaction causes a low-purity product as a result of the attack of methanol on the glycosyl fluoride, giving rise to methyl cellobioside as by-product. The reaction time for the neutralization by using the ionexchange resin should also be as short as possible since the longer treatment by an acid leads to the decomposition or anomerization of β -cellobiosyl fluoride.

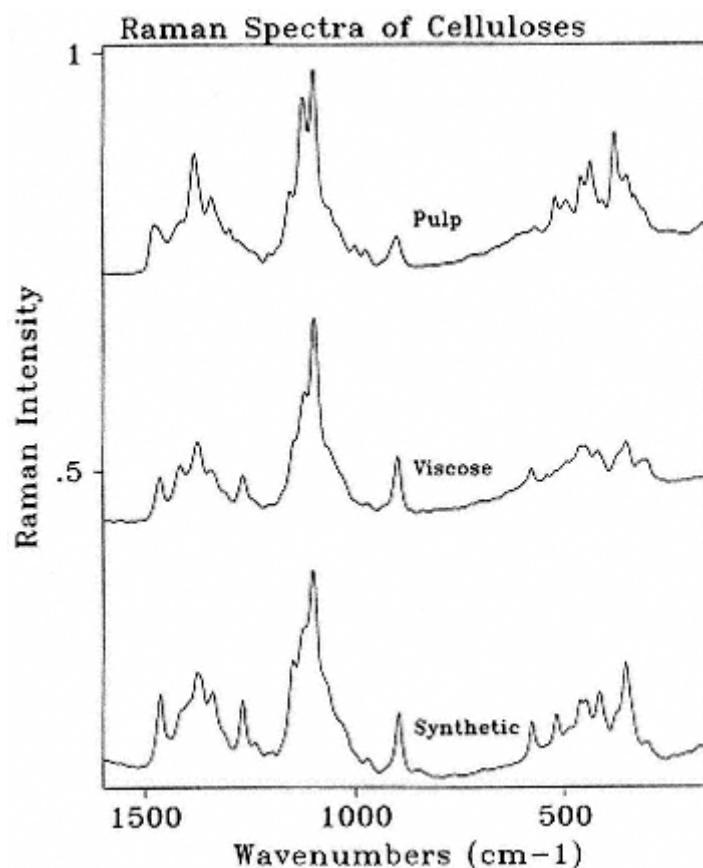


Fig. 8.
FT-Raman spectra of pulp, viscose, and synthetic cellulose.

6. ^1H NMR (D_2O) for β -cellobiosyl fluoride: δ 5.17 ppm (dd, $J_{\text{IF}} = 53.0$, $J_{\text{IF}} = 7.25$ Hz, 1H, anomeric proton of reducing end unit), ^{19}F NMR ($\text{D}_2\text{O} + \text{C}_6\text{F}_6$) δ -8.4 ppm (dd, $J_{\text{IF}} = 53.0$, $J_{2\text{F}} = 14.1$ Hz).

7. The β -cellobiosyl fluoride should be stored in the refrigerator (4°C) to avoid the anomerization to α -cellobiosyl fluoride.

8. In order to avoid the nonenzymatic hydrolysis of β -cellobiosyl fluoride monomer, acetonitrile should be added and the polymerization should be started by the addition of enzyme immediately after the buffer solution of β -cellobiosyl fluoride is prepared.

9. As the enzymatic polymerization proceeds, the initially homogeneous solution gradually becomes heterogeneous with a white precipitation of the product polysaccharide.

10. The fluorine atom at the polymer end is converted into a hydroxyl group during the inactivating procedures.

11. As a result of pouring the reaction mixture into methanol/water, the catalyst cellulase, lower molecular-weight products like glucose and cellobiose, and a trace amount of inorganic compounds derived from the buffer solution are removed.

12. ^{13}C NMR spectrum of water-insoluble part (D_2O) δ 62.2 (C6), 72.8–76.6 (C2,3,5), 87.6–88.7 (C4), 104–107.1 (C1).

13. ^{13}C NMR spectrum of the water-soluble part (D_2O) δ 60.7 (C6), 61.4 (C6 of nonreducing end unit), 70.3 (C4 of non-reducing end unit), 71.0 (C5 of reducing end unit, α -form), 72.1 (C2 and C3 of reducing end unit) 73.8 (C2), 74.0 (C2 of nonreducing end unit) 74.8 (C3), 75.6 (C5 of reducing end unit, β form), 76.3 (C3 of non-reducing end unit), 76.8 (C5 of non-reducing end unit), 79.2 (C4), 92.7 (C1 of reducing end unit, α form), 96.6 (C1 of reducing end unit, β form), 103.2 (C1), and 103.4 (C1 of nonreducing end unit).

14. Spectra can be recorded at a number of resolutions. In the present work spectra are recorded at two resolutions of 4 and 2 cm^{-1} . While this does not improve the band profiles for the IR spectra, it does sharpen the Raman spectra by a small but noticeable amount. Thus, the Raman spectra presented are recorded at 2 cm^{-1} and the IR spectra at 4 cm^{-1} resolution.

15. The number of scans chosen for the FT-IR and FT-Raman measurements is based on obtaining satisfactorily high signal to noise ratios in the resultant spectra. Note that at higher resolutions more scans are required to generate a given signal to noise ratio.

16. The advantage of using the IR microscope in this analysis is that the sample is not affected by any adverse sample preparation. This contrasts with the KBr disc method where the sample needs to be finely ground and exposed to high pressures. Also, KBr discs are prone to be contaminated with impurities, particularly water. Thus, the IR microscope measurement produces the IR spectrum of solely the pristine sample.

17. To obtain high-quality IR microscope spectra of celluloses, it is important to measure the spectra through suitably thin sample areas. In the context of cellulose, which is a very strong IR absorber, it is often difficult to “thin” the sample to the few microns required for the optimum absorbances. In order to realize the full IR spectroscopic detail of the IR spectrum, one should strive for a maximum absorbance in the range of one. Thus spectra of more typical “thick” cellulose samples are not presented here. Obtaining the optimum cellulose spectrum is essentially done by a trial and error process, until a suitably thin measurement area is located. When necessary samples may be thinned by gently rolling with, for example, a Spectra-Tech metal rolling device (Nicolet Ltd, Warwick, Warwickshire, UK).

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6—

Modification of Alginate Using Mannuronan C-5-Epimerases

Helga Ertesvåg and Gudmund Skjåk-Bræk

1—

Introduction

The enzymatic interconversions of the various hexoses found in polysaccharides and glycoproteins normally take place either on the unsubstituted sugars, their phosphate esters, or at the sugar nucleotide levels. In a few cases, however, epimerization reactions take place after the polysaccharides are formed. This type of postpolymerization epimerization is a feature of several uronic-acid containing polysaccharides found both in eukaryotes and in bacteria. These reactions are exemplified by C-5 inversion of D-glucuronic acid into L-iduronic acid in dermatan **(1)** and heparin **(2)** synthesis and D-mannuronic acid (M) into L-guluronic acid (G) in alginates **(3)**. The latter epimerization (**Fig. 1**), which is catalyzed by mannuronan C-5 epimerases is the last step in the biosynthesis of alginates. Since M prefers the 4C_1 -conformation, while G prefers the 1C_4 -conformation, the relative amount and distribution of each monomer has a profound effect on the secondary structure and thus on the properties of the polymers **(4)**. Although stretches of consecutive M (M blocks) or alternating M and G (MG blocks) are relatively flexible, stretches of consecutive G (G blocks) are fairly stiff. Furthermore, the gelforming properties are related mainly to the content and sequential arrangements of guluronic acid. Two contiguous, diaxially linked G-residues form binding sites for calcium ions, and long sequences of such calcium-binding sites form crosslinks with similar sequences in other alginate molecules, giving rise to junctions in the gel network. It has also been found that long M blocks are immunogenic **(5)**.

By treating alginate with the enzyme mannuronan-C-5 epimerase, it is possible to alter both the flexibility and the gel-forming and immunogenic

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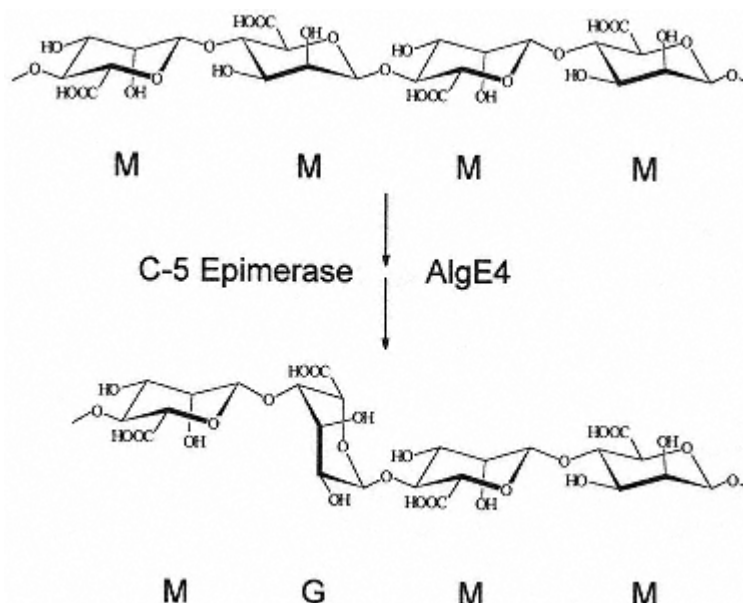


Fig. 1.

Epimerization of M to G in alginate by the enzyme AlgE4.

properties of the polymer. This enzyme thus determines the properties of the produced alginate. Mannuronan C-5 epimerase is identified in a range of brown algae (6,7) as well as in alginate-producing bacteria, such as *Pseudomonas aeruginosa* and *Azotobacter vinelandii* (3,8). Both *P. aeruginosa* and *A. vinelandii* contain a periplasmic C-5 epimerase encoded by the *algG* gene (8,9). In addition *A. vinelandii* has recently been shown to encode a family of secreted, calcium-dependent epimerases (AlgE1-5) (10). These epimerases have been cloned and expressed in *Escherichia coli*, and it has been shown that they can be used to modify any alginate in vitro (11). Even though the AlgE epimerases are homologous, their products are not identical. It has been shown that although AlgE2 predominantly introduces G as G blocks, the end product of AlgE4 is an alginate with only M and MG blocks (10). Thus, it is possible to increase the amount of MG and/or G blocks in any alginate by using these enzymes or a mixture of them.

Since these enzymes are not commercially available yet, they have to be expressed recombinantly and purified as described in **Subheading 3.3**. The activity-assay (**Subheading 3.2**) is based on the findings that the enzymic activity is accompanied by an exchange of proton at C-5 with the medium (12). By using a [5-³H]-labeled mannuronan as substrate, the activity is measured as tritium released into the water. The preparation of the sub-

strate for this assay is described in **Subheading 3.1**. The purified enzymes can then be used for modification of the alginate as described in **Subheading 3.4**. Before the epimerized alginate is used, it should be analyzed by NMR spectroscopy (**13,14**) to find the new composition of the sample as outlined in **Subheading 3.5**.

2—

Materials.

2.1—

Preparation of [5-³H]Polymannuronic Acid

1. Liquid medium for *P. aeruginosa*: (20 g) sodium D-gluconate, (20 g) sodium D-gluconate, (20 g) sodium glutamate, (3 g) Na₂HPO₄, (0.3 g) MgSO₄ • 7 H₂O dissolved in 1 L distilled water. Sterilize by autoclaving.

2. Agar medium: (20 g) Trypton (Difco Laboratories), (10 g) yeast extract (Difco), (10 g) NaCl, (2 g) and glucose, (30 g) agar (Difco) dissolved in 2 L distilled water and autoclaved for 20 min. at 120°C.

3. [5-³H] glucose (25 µCi/plate 15.7 Ci/mmol; Amersham).

4. NaOH.

5. Dilute HCl.

6. NaCl.

7. 96% Ethanol.

8. 70% Ethanol.

9. Ethylether.

10. 1.2-, 0.7-, and 0.2-µm filter (Sartorius).

2.2—

Epimerase Assay

1. 0.5 M MOPS, pH 6.9: store at 4°C.

2. MC-buffer: 20 mM MOPS, pH 6.9, 2.2 mM CaCl₂.

3. Isopropanol.

4. 5 M NaCl.

5. Scintillation Cocktail (Beckman Ready Safe).

2.3—

Purification of Epimerase

1. 0.2-µm filter, preferably with a prefilter.

2. HiTrapQ column (Pharmacia).

3. 3X LB: 30 g trypton, 15 g yeast extract, 5 g NaCl, distilled water to 1 L. Sterilize by autoclaving.
4. MC buffer with and without 1 M NaCl.
5. Isopropyl β -D-galactopyraoside (IPTG) (Sigma, St. Louis, MO).

2.4—

Epimerization of Alginate

1. 50 mM MOPS (pH 6.9).
2. 0.1 M CaCl₂.
3. 0.5 M EDTA pH 8.0.
4. Dialysis tubing, MWCO 12–14000.

2.5—

Analysis of Alginate by NMR Spectroscopy

1. 0.01 M HCl.
2. 0.01 M NaOH.
3. D₂O.
4. 0.3 M Triethylene tetraamine hexaacetate (TTHA) dissolved in D₂O.
5. 1% Trimethylsilyl propionic-2,2,3,3diacid sodium salt.

3—

Methods

3.1—

Preparation of [5-³H]Polymannuronic Acid

1. Grow *P. aeruginosa* in the liquid medium for 3 d at 32°C.
2. Plate out 0.5 mL on each of six agar plates (diameter 14.5 cm) and incubate at 20°C for 24 h. Add [5-³H] glucose (25 µCi/plate, 15.7 Ci/mmol; Amersham) and incubate the plates for another 3 d.
3. Scrape off the cells and exopolymer with a rubber policeman, transfer to 400 mL 0.1 M NaCl and stir for 1 h.
4. Remove the cells by centrifugation and filter the supernatant through 1.2, 0.7, and 0.2 µm (Sartorius) subsequently.
5. NaOH should be added to a final concentration of 0.1 M to deacetylate the polymer for 30 min at 22°C.
6. Adjust the pH to 7.0 by diluted HCl and dialyze the polymer solution, 2 × 24 h, against MQ water.
7. Reduce the volume to half on a rotavapor, and add NaCl to 0.2% w/v.
8. Precipitate the radiolabel polymer by adding an equal volume of 96% ethanol, and collect the polymer by spinning it onto a glass rod.
9. Wash the polymer twice with 70% ethanol, 96% ethanol, and finally ethylether. After drying, dissolve the pellet in distilled water (0.25%) and filter the solution as described above (**Step 4**) and freeze-dry.

3.2—

Epimerase Assay

1. Dissolve the tritiated alginate in water (2 mg/mL). This takes some time, but is usually achieved by shaking overnight. Measure the specific activity in a β-counter (see Note [1](#)). Freeze aliquots of the solution in 1.5-mL tubes at -20°C.
2. Mix 540 µL MC buffer, 10 µL enzyme, and 50 µL alginate in a 1.5-mL tube. This gives a final concentration of calcium of 2 mM (see Note [2](#)).

3. Incubate at 37°C for 1 h (see Note [3](#)).
4. Precipitate the alginate by adding 15 µL 5 M NaCl and 800 µL isopropanol, and freeze at -50°C or lower for more than 15 min (see Note [4](#)).
5. Centrifuge at 15,000g (or more) for 30 min.
6. Transfer 1 mL of the supernatant to a vial for scintillation counting. Take care not to disturb the pellet.
7. Add a scintillation cocktail for water-containing solutions.
8. Measure the DPM in the sample.

3.3—

Purification of Epimerase (see 5).

1. Grow an *E. coli* containing a plasmid encoding the epimerase of interest overnight in 3X LB containing the appropriate antibiotic at 37°C with shaking.
2. Inoculate 1% in new, prewarmed 3X LB, and continue incubation. One liter usually gives a lot of enzyme.
3. Induce with IPTG after 3 h to a final concentration of 0.5 mM.
4. Harvest the cells by centrifugation (4000g for 5 min) after about four additional hours (see Note 6).
5. Resuspend the cells in 1/10 volume of chilled MC buffer, work on ice from this step onward.
6. Disrupt the cells by sonication; the conditions have to be set for each sonicator (see Note 7).
7. Centrifuge the sonicated solution at 27,000g for 30 min.
8. Filter the supernatant through a 0.2-μm filter (it is easier if the filter has a prefilter, too) (see Note 8).
9. Apply the filtrate to a HiTrap Q column equilibrated with MC buffer. A 5 mL column can absorb epimerase from up to 50 mL filtrate.
10. Elute the epimerase using MC buffer with NaCl. (A continuous gradient works best). The epimerases is eluted between 0.3 and 0.6 M NaCl depending on which epimerase is being purified.
11. Analyze the interesting fractions for activity.

3.4—

Epimerization of Alginate

Estimate the epimerization obtained by the enzyme preparation:

$$\frac{(\text{Measured activity} - \text{blank}) \times \text{enzyme (mL)} \times 1.4 \times 100\%}{\text{enzyme} \times \text{h added activity} \times \text{incubation-time (h)}} = \% \text{ epimerization/mL}$$

Determine how much alginate you want to epimerize and which G content you want to obtain. As shown in Notes 2 and 3, **you can then find a combination of time and amount of enzyme, which will give the wanted epimerization. Epimerize a small batch of the alginate first using the calculated time and amount of enzyme to ensure that the enzyme behaves as expected on the new substrate:**

1. Dissolve 7.5 mg alginate in 1 mL H₂O.
2. Add 5 mL of a prewarmed mixture of 50 mM MOPS and enzyme containing 2.4 mM calcium.
3. Incubate at 37°C for the calculated time.
4. Stop the reaction by adding EDTA to 10 mM.

5. Dialyze the solution against deionized water. It is important that all calcium is removed before NMR spectroscopy.
6. Analyze the alginate by NMR spectroscopy (see **Subheading 3.5.**).
7. Use the results from the NMR analysis to adjust the reaction-conditions before scaling up (see Note [9](#)).

3.5—

Analysis of Alginate by NMR Spectroscopy

Preparation of samples (to obtain a sufficiently resolved NMR spectrum of alginate, it is essential to depolymerize the sample slightly to reduce the viscosity):

1. Dissolve 20 mg alginate in 20 mL distilled water, adjust pH to 5.6 with 0.01 M HCl, and hydrolyze the sample for 1 h at 100°C.
2. Cool the sample and adjust the pH to 3.8, and hydrolyze at the same conditions for another 30 min.
3. Cool the sample, and neutralize to pH 6.8 with 0.01 M NaOH, and freeze-dry.
4. Dissolve 7.5 mg freeze-dried material in 700 μL D_2O .
5. Add 20 μL 0.3 M Triethylen tetraaminehexaacetate (TTHA dissolved in D_2O) (see Note [10](#)) and 5 μL 1% trimethylsilyl propionic-2,2,3,3, diacid sodium salt. Record the ^1H -NMR spectra at 90°C on a 100–500 MHz spectrometer (see Note [11](#)).

4—

Notes

1. When 50 μL of this alginate solution are used later in the procedure, it is supposed that this amount contains about 10,000 dpm. If the activity is much less, more alginate must be used, or lower counts must be acceptable. (If the object is to find out which column fractions contain the epimerase, you do not need exact results.) If the volume of the alginate solution is increased, the concentration of calcium in the buffer has to be increased too.
2. 2 mM of calcium are sufficient for all tested epimerases, although some need more for optimal activity. If the concentration of calcium is increased much above 4 mM, the alginate might start to gel and be less accessible to the enzyme, especially if the enzyme used introduces G-blocks. When about 25–30% of the substrate is epimerized, the reaction-rate slows down. This is most pronounced for enzymes making MG-blocks. Thus, if you obtain DPM of more than 3,000–4,000, the reaction should to be repeated using less enzyme or less time (see Note [3](#)).
3. The time can be varied from 1 min to several days. The enzymes are stable at 37°C. If <1 h is used, the buffer and alginate should be prewarmed to avoid effects of the lower rate at lower temperatures.
4. Alternatively freeze at -20°C for about an hour. The reaction can be stored in the freezer for weeks.
5. This procedure removes more than 90% of the contaminating proteins. The epimerases do not need to be pure to be used in subsequent epimerizations. If the amount of protein in the epimerized alginate is a problem, further purification can be achieved by using a hydrophobic interaction column (Pharmacia HiTrap Phenyl Sepharose High Performance has been used in our laboratory; 0.75 M $[\text{NH}_4]_2\text{SO}_4$ is a useful start condition; it can be adjusted somewhat up or down to obtain optimal separation). Be aware that while some AlgE epimerases increase the activity in the presence of $(\text{NH}_4)_2\text{SO}_4$, others show a decreased activity. We have found that *E. coli* seems to make several forms of the enzymes, since several bands can be seen on an SDS-PAGE gel. The largest (and probably most like the native) enzyme has an apparent molecular weight of about 20 kDa more

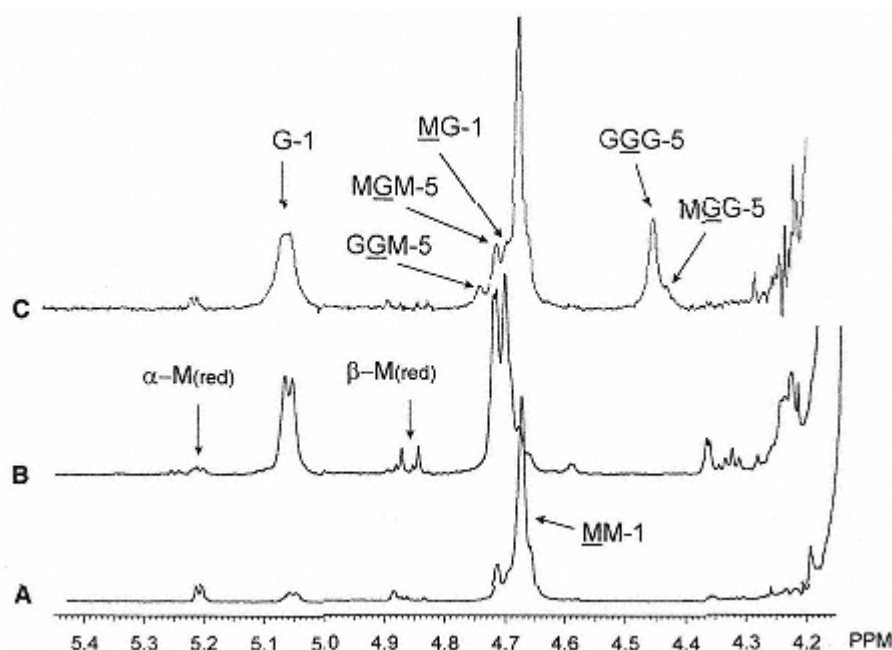


Fig. 2.

Assignment of the peaks in ^1H -NMR spectra. (A) Alginate with 5% G, (B) alginate epimerized by AlgE4, (C) alginate epimerized by AlgE2.

than that deduced from the DNA-sequence, the smallest band have an apparent molecular weight close to the one expected from the DNA sequence. This is of no importance when the object is to epimerize alginate, but it does make the protein-preparation look less pure than it really is.

6. The amount of epimerase per cell increases toward the end of the exponential phase, but decreases markedly when the culture reaches the stationary phase. The times given in the procedure usually give good results.

7. The optimal conditions can be found by varying pulse time, power output, and overall time, and determining what gives the highest activity. It is important to use several small pulses and to keep the sample on ice to avoid warming it up.

8. The filtered enzyme solution can be kept refrigerated for a day or two without losing activity.

9. When a large-scale epimerization is performed, the concentration of alginate is dependent on the polymer length (which determines the viscosity of the solution). A 0.1–0.2% solution is usually acceptable.

10. TTHA dissolved in D_2O serves as a sequestrant for divalent cations, i.e., to avoid the formation of aggregates.

11. ^1H -NMR spectra of alginate is given in **Fig. 2**. For further assignment of the alginate spectrum and for quantitative determination of the sequential structure, see **refs. 13** and **14**.

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7—

Viscosity Control of Guar Polysaccharide Solutions by Treatment with Galactose Oxidase and Catalase Enzymes

Michael J. Donnelly

1—

Introduction

Guar gum is a natural polysaccharide found in the seeds of certain annual leguminous plants (*Cyamopsis psoraloides* and *tetragonalobus*). In a series of physical operations involving crushing, sifting, and grinding, the seeds are separated from the pod and then the gum, contained in the endosperm, is separated from the rest of the seed. These processes influence the properties of the polymer, such as the molecular weight and purity, which in turn affect the viscosity of water solutions of the polymer. The variability of availability and composition of the gum is further underlined by the fact that part of the world production derives from Third World countries. These often utilize manual labour to harvest the crop and are in geographical regions subjected to wide fluctuations in climate.

The guar polymer is an alternating copolymer composed of a mannose backbone (linked β 1–4) with evenly spaced single galactose units (linked α 1–6) as sidechains attached to each alternate mannose unit. A representation of the structure of guar is given in **Fig. 1**.

Aqueous solutions of the polymer find numerous and diverse applications in both food and industrial areas in the Western world based on their suspending and thickening capabilities. Although guar is one of the most effective viscosifying polysaccharides known, the range of available viscosities has been extended to meet these diverse industrial needs (**1**). This is achieved by chemical derivatization of guar, for example, via esterification or etherification. Alternatively, chemical additives may be used to control the rate, effectiveness, and resulting viscosity of guar solutions. In the latter case, reagents, such

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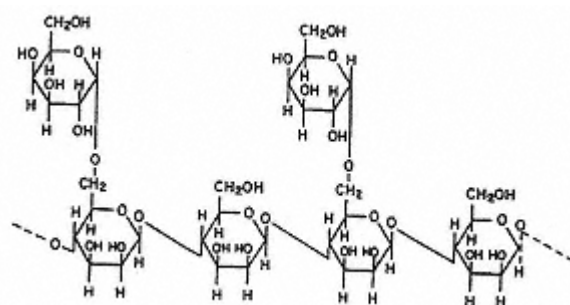


Fig. 1.

Representation of the structure of guar polysaccharide.

as borax or transition metals, are used, both of which take advantage of the *cis*-hydroxyl groups present in the guar structure to form complexes. Combinations of derivatization and additives can be used, but generally this can lead to reduced viscosity enhancement. Some of these routes also have other disadvantages. For example, the exclusion of use of transition metal complexes in food areas and the possibility of precipitation or unstable gels being formed when polyvalent cations are utilized for viscosity increase.

As an alternative to these procedures, the following method utilized a dualenzyme system that can be used to produce various viscosities or gels under mild conditions with low toxicity reagents. The steps in the procedure are as follows:

1. Pasteurization and drying of guar powder to improve the microbiological stability of the polymer.
2. Preparation of a dilute solution and adjustment of reaction conditions of temperature and pH to the optimum for the enzymes. Solution can be prepared in either pH-adjusted water or in buffer solution.
3. Addition of enzymes and incubation to reach the desired viscosity or gel formation.
4. Use of the viscous solution or gel, or isolation of modified guar powder for later application following redissolution.

Studies on the enzyme galactose oxidase show it is able to oxidize specifically the hydroxyl group at the C-6 position in galactose. The enzyme seems capable of this when the galactose is contained in certain short-chain and in some polymeric carbohydrates (2,3). Guar is no exception, and it is presumed that the galactose unit is sufficiently accessible in this polymer for the enzyme to be catalytically active. Mechanistic studies on the action of the enzyme on methyl galactoside and oligosaccharides containing the D-galactopyranosyl residue (4,5) indicate that the C-6 hydroxymethyl group is converted to an aldehyde. This can then be ultimately converted to a carboxylic acid (Fig. 2). A by-product of the former reaction is the generation of hydrogen peroxide, which is inhibitory to the enzyme. Catalase enzyme is therefore also added to

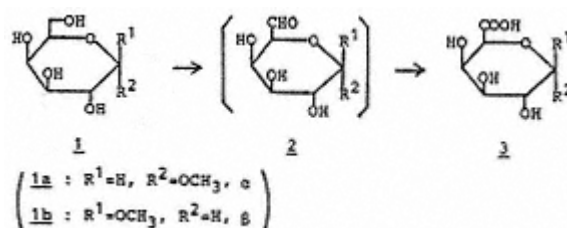


Fig. 2.
Enzymic conversion of C6 hydroxyl to carboxylic acid.

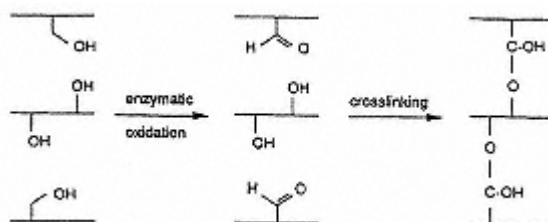


Fig. 3.
Proposed mechanism for crosslinking reaction.

the reaction mixture to convert the hydrogen peroxide into noninhibitory oxygen and water. For the case of modification of guar solutions with galactose oxidase/catalase, large viscosity increases can occur even up to complete gelation of the reaction mixture **(6)**. This behavior is characteristic of a crosslinked polymer. It can therefore be postulated that the intermediate aldehyde group produced at the C-6 position could form a hemi-acetal group by reaction with a distant hydroxyl group of the same polymer chain (ring formation by a backbone-biting mechanism), or with a hydroxyl group on a different polymer chain (interchain crosslinking mechanism). These concepts are illustrated in **Fig. 3**, and both can contribute to the observed viscosity increase and ultimate gelation of the guar solution **(6,7)**. Greater increases in viscosity can be achieved by reaction in buffer solution, and it is assumed the buffer minimizes adverse effects of any uronic acid formation, such as lowering of the pH to a region that is not optimum for the enzymes used. A contribution to the viscosifying effect from intra- and interchain ester formation between uronic acid and hydroxyl groups may also occur. This, however, is probably limited owing to the high water concentration of the reaction system driving the equilibrium in the reverse direction to ester synthesis.

2—

Materials

1. Galactose oxidase (D-galactose: oxygen 6-oxidoreductase; EC 1.1.3.9) enzyme from *Dactylium dendroides* is a commercially available lyophilized powder con-

taining a defined number of units of activity per weight of powder (obtained from Sigma Chemicals, St Louis, MO, or Worthington Biochemicals Corp., Freehold, NJ). This is stored at -20°C in a screw-cap bottle, which has been sealed in an air- and moisture-tight plastic bag until ready for rehydration. One unit of activity is defined as that which will produce an absorption change at 425 nm of 1.00/min at pH 6.0 and 25°C using a peroxidase *o*-tolidine (0.5% w/v *o*-tolidine in methanol) system and galactose (10 % w/v in distilled water) as substrate (3.4-mL reaction volume, 1-cm light path) (see **Note 1**).

2. Guar powder is stored in a previously heat-sterilized (110°C for 16 h) moisture-tight screw-cap bottle until used. All glassware used is sterilized under these conditions (see **Notes 2 and 3**).

3. 0.1 M Phosphate buffer, pH 6.0. Mix 5.8 mL of 0.2 M potassium hydroxide solution and 50 mL of 0.2 M potassium dihydrogen phosphate solution together with 44.2 mL distilled water/100 mL of buffer solution. Based on the total weight of buffer solution, 0.1 % w/w sodium benzoate is added and dissolved by shaking (see **Note 4**). **The solution is heated to 90°C for 3 min, then removed from the heat, covered with sterile glassware, and allowed to cool to ambient before use. The same procedure for addition of preservative and heating is used for the distilled water used for the enzymic reactions, but in this case, the pH is initially adjusted to 6.0 with 0.1 M hydrochloric acid.**

4. Catalase (twice crystallized from bovine liver (Sigma) containing approx 44,000 U/mg of protein) is stored in a clean sealed plastic bottle in the refrigerator (5°C) until used.

3—

Methods

All actions are carried out as far as possible in a fume cupboard to minimize exposure to chemicals, solvent vapor or polysaccharide dust. Handling of powders is also carried out wearing a suitable dust mask.

3.1—

Pasteurization and Drying of Guar Powder.

1. Guar powder (125 g) is slurried and thoroughly wetted out in a round-bottom flask containing 500 mL of an 80:20 2-propanol:water mixture (see **Note 5**). **The flask is fitted with a stirring gland and water-cooled condenser. Agitation is achieved from use of an electric motor driving a wide half-moonshaped polytetrafluoroethylene stirrer that fits close to the internal dimensions of the flask.**

2. The slurry is heated to reflux (~ 82°C) using a water bath or electric heater over about 15 min and under agitation at approx 50 rpm. Reflux conditions are maintained for 10 min.

3. The flask is removed from the heater, the neck covered with a heat-sterilised Petri dish, and then allowed to cool to about 70°C.

4. The cover is removed and the slurry is filtered under suction at about this temperature using a grade 2 glass sinter protected by two filter papers (grade 540). Apart from addition of more slurry, the sinter is covered with the Petri dish

throughout the operation. A white fluffy powder is collected in the sinter and a pale-yellow filtrate results.

5. While still covered with the Petri dish, the solid is further dried by allowing air to pass over it for about 10 min.

6. The damp cake is transferred to a preweighed Petri dish and placed in a vacuum oven preheated to 60°C. Vacuum is applied, and the solid dried to constant weight over approx 14 h (see Note [6](#)).

7. The dried solid is then transferred to a container for storage. Recovery of alcohol-treated guar is about 94%.

3.2—

Preparation of Dilute Solutions and Control of Reaction Conditions

Dilute solutions of guar (1% w/w) are made up in pH 6.0 distilled water or pH 6.0 phosphate buffer as follows.

1. A volume of one of these liquids (396 mL) is placed in a 600-mL squat beaker.

2. A heat-sterilized metal propeller blade (45-mm diameter) is immediately placed in the liquid, set within a few mm of the base of the beaker, and coupled to a highspeed agitator.

3. The beaker is then covered with a clean filter paper, which has a slit and small central hole cut in it to contain the shaft of the agitator. The filter paper is held in place by folding the edges down onto the lip of the beaker. Alcohol-treated guar (4.00 g) is weighed into a beaker and covered with filter paper.

4. The agitator is set in motion at approx 1600 rpm providing a deep vortex. The slit in the filter paper covering the liquid-containing beaker is parted slightly, and the guar powder steadily added to the slope of the vortex over about 2 to 3 min (see Note [7](#)). The filter paper is replaced in its original position around the shaft and agitation continued for about 5 min. The agitator is switched off to check the dispersion of powder is even and then agitation is continued for a further 15 min.

5. The agitator is stopped and removed, and the beaker covered with a clean plastic bag and kept at ambient temperature for 24 h.

6. The viscosity of a portion of the solution is then measured using a cone and plate viscometer at a shear rate of 1.92 s⁻¹.

3.3—

Enzyme Reaction to Increase Viscosity

1. Galactose oxidase solutions are made up immediately prior to use by dispersing an accurately weighed (4 decimal place balance) quantity of the lyophilized powder into a volume of cold (5°C) 0.1 M, pH 6.0, potassium phosphate buffer. The suspension is mixed by gentle swirling by hand to form a solution that is stored in the refrigerator (5°C) before use (maximum ~30 min) (see Note [8](#)).

2. A known volume of guar solution is transferred aseptically (near the updraft of a bunsen flame) into a baffled shake-flask and sealed with a heat-sterilized rubber bung.

3. A volume of galactose oxidase solution containing a known number of units of activity (per 100 mL of 1% guar solution) is added to the solution and the flask,

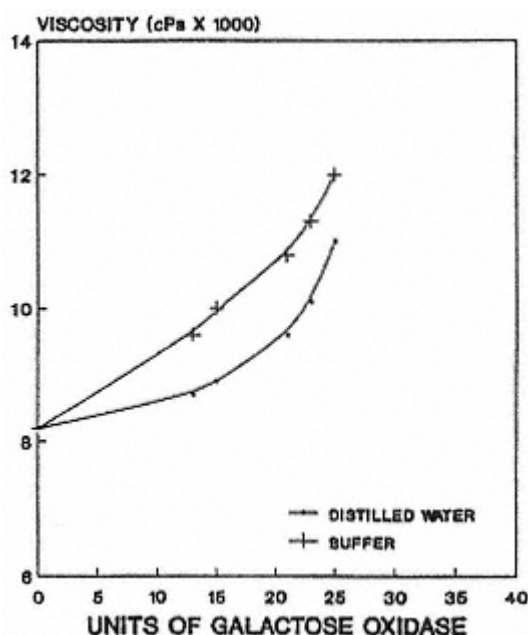


Fig. 4.
Viscosities achievable using low levels of galactose oxidase.

after resealing with a heat-sterilized rubber bung, is rapidly swirled by hand to disperse the enzyme.

4. After removal of the bung, catalase is added by micropipetter (20 μ L/100 mL of 1% guar solution, about 80,000 U), the flask is resealed with the bung, and the solution is swirled rapidly by hand to disperse the enzyme (see Note 9).

5. The rubber bung is replaced with a heat-sterilized flexible polyurethane foam bung and covered with sterile grease-proof paper (see Note 10). The flask is then placed in a preheated (28°C) orbital incubator (diameter of orbit 32mm), which is operated at 200 rpm for a set period of time.

Fig. 4 shows the viscosity increases that are achieved, in either pH 6.0 distilled water or pH 6.0 phosphate buffer, by the addition of a low level of galactose oxidase (between 10 and 30 U/100 mL of 1% guar solution; 20 μ L catalase added in each case) and after incubation for 40 h. The viscosities are measured at a shear rate of 1.92 s^{-1} using a cone and plate viscometer, and this also applies to the data of **Figs. 5** and **6**. The data of **Fig. 5** show that the type of rheology exhibited by guar is not altered by enzymic treatment. Both untreated and enzyme-treated polymer solutions show, over the shear rates investigated, similar shear thinning behavior, but the enzyme-treated material does so at higher viscosity values. **Figure 6** shows an example of the higher viscosi-

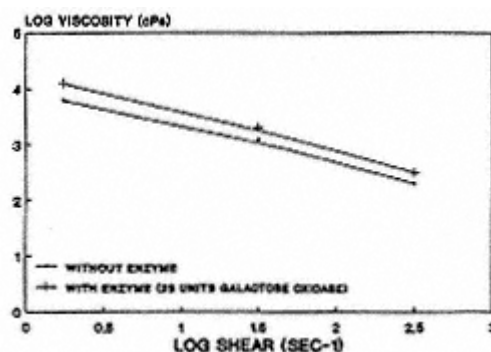


Fig. 5.
Rheology comparison of enzyme-modified
and unmodified guar solutions.

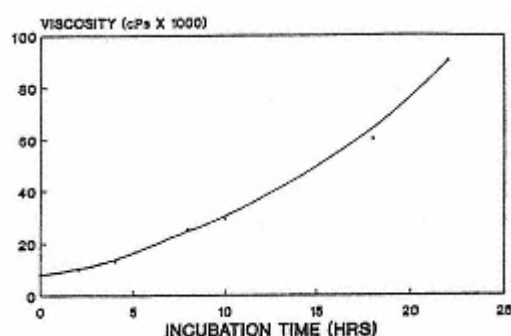


Fig. 6.
Viscosities achievable with high levels of
galactose oxidase.

ties that can be achieved when a high dose of galactose oxidase is used (125 U plus 20 μ L catalase/100 mL of 1% guar solution made up in pH 6.0 phosphate buffer). In this case, the incubation time is varied between 2.5 and about 22 hr. Complete gellation of the mixture takes place shortly after the later reaction period as indicated by the reaction mixture forming a single entity.

3.4—

Isolation and Redissolving of Modified Guar Product

1. After the desired viscosity is achieved, the modified guar solution or gel is preferably used directly. Alternatively, the polymer may be isolated and dried for later use. Isolation is achieved by transferring the contents of the reaction flask into a beaker containing 2-propanol. The volume of alcohol used is twice the weight of the contents of the flask. The precipitate is broken up with a spatula and then stirred vigorously with a propeller-shaped agitator driven by an electric motor for about 15 min. Occasionally, the motor is turned off and the precipitate further broken up with a spatula (see **Note 11**).

2. The suspension is then filtered through a glass fiber disk (Whatman grade G/FC) under vacuum, the disk being supported on a ceramic Buchner filter.
3. After removal of the vacuum, the collected mass is carefully eased off the disk and slurried with 2-propanol (15 mL) using a spatula and the vacuum reapplied.
4. This washing stage is repeated once more.
5. The isolated solid is removed from the filter disk and placed in a preweighed Petri dish and dried to constant weight in a vacuum oven at 55°C for typically about 5 h on this scale.
6. The friable material is then ground to a fine powder using a pestle and mortar, and stored in a heat-sterilized container.

Dissolution of the modified guar is carried out using the same procedure as for making up the original guar solutions but with longer rehydration periods being used for higher viscosity or gelled products (see **Note** [12](#)).

4— Notes

1. Galactose oxidase is required to be stored at low temperature and under dry conditions to maintain stability and activity. As an extra precaution against atmospheric moisture condensing as ice around the exterior and neck of the container, the container is protected by a moisture and air-tight plastic bag.
2. Guar powder needs to be protected from atmospheric moisture during storage. Ingress of water can form lumps and gels, which makes the powder heterogeneous and difficult to disperse and dissolve completely. The presence of moisture can also increase or initiate microbial growth or enzymic action leading to deterioration of the polymer structure.
3. All glassware and storage containers are heat-sterilized in order to minimize the risk of microbial contamination of guar powders, solutions, or products.
4. Sodium benzoate is added to improve the solution stability of alcohol-treated guar further. Solutions prepared without the addition of this material do not reach the same maximum viscosity during the 24-h hydration period and may, for example, be ~15% lower than solutions with added benzoate. End users of guar solutions can expect to have completed the preparation and utilization of the solutions over time scales of a maximum of a few days. Over this sort of period (up to 3 d), benzoate-free solutions can show a typical drop of 20% in viscosity, but addition of benzoate produces a virtually viscosity stable solution with reductions only of the order of 2–3% at 25°C.
5. Propanol-water mixtures must be made up **before** addition of the guar powder. Addition of water separately either before or after the alcohol would lead to lump and gel formation, and an inability to disperse the polymer correctly. Great care must be taken to wet-out the powder completely with the alcohol-water mixture and for the slurry to be evenly dispersed during the Pasteurization step. Guar solution prepared without Pasteurization reaches a much lower maximum viscosity than benzoate-free material. Often guar powder is so heavily contaminated with microbes and enzymes that material that has not been Pasteurized forms solutions that are stable for only a few hours before viscosity is reduced to near-zero values.

6. The maximum temperature during drying of guar is $\sim 60^{\circ}\text{C}$ in order to minimize decomposition and coloring of the polymer.

7. Addition of powder during preparation of dilute solutions of guar needs to be carried out carefully and slowly. Too fast an addition will lead to clumping and lump formation, making it impossible to generate homogenous solutions.

8. Galactose oxidase solutions should be made up as near to the time of use as possible. Solutions can be made up and frozen, but repeated freezing and thawing can affect the activity of the enzyme. Too vigorous agitation during the dispersion of the freeze-dried powder should also be avoided in order to minimize frothing and possible deactivation of the enzyme.

9. A high number of units of catalase enzyme are added, since this is a cheap enzyme and needs to be present at a concentration that ensures complete removal of inhibition of galactose oxidase by hydrogen peroxide.

10. During enzymic reaction, the baffled shake flasks must be sealed only with a flexible polyurethane foam bung. Complete sealing with a rubber bung during this stage must be avoided, since if the Pasteurization step has been incomplete, then microbial action can lead to gaseous product formation, buildup of pressure in the flask, and possible explosion.

11. If after enzymic reaction, low or modest viscosity products have been formed and precipitated for later use, then the rehydration time scales are similar to those for the original guar powder. If, however, very high viscosity products have been formed and the material precipitated for later use, then an extended rehydration period must be allowed to recover the viscosity fully. When reaction has been carried out to form a gel, this can be broken down using a high shear mixer and, after precipitation and drying, the powder is ground mechanically. All of these aid the rate of rehydration, but extended rehydration periods may still then also be required for these highly crosslinked materials. The time scales are dependent on the mixing equipment used, but are typically twice that for untreated guar.

12. Other permutations of reaction time and units of galactose oxidase used are possible to achieve desired viscosities. Polymer concentrations can also be increased provided suitable equipment is available to mix the viscous solutions efficiently. Polymer concentration as high as 50% can be used, at which point guar has a crumb-like consistency, but this requires specialist equipment for mixing such as z-blade mixers. The procedure for increasing the viscosity of guar can also be applied to other galactomannans, such as locust bean gum.

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The Production of Cyclodextrins Using CGTase from *Bacillus macerans**

Jacob A. Rendleman, Jr.

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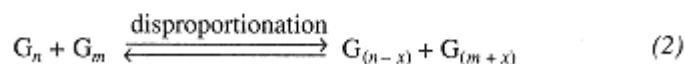
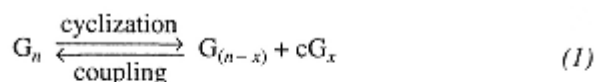
Introduction

Cyclodextrins (cyclomaltooligosaccharides, cyclic [1→4]- α -D-glucans, CDs) are produced by the action of cyclodextrin glucanotransferase (CGTase) on either liquefied starch, maltodextrin, or long-chain maltooligosaccharides. Although numerous bacterial sources for the production of the enzyme are known, the CGTase employed in the reactions described in this chapter is derived from cultures of *Bacillus macerans* and is available commercially. In the absence of compounds capable of forming inclusion compounds (complexes) with CDs, conversion reactions with *B. macerans* CGTase normally favor the formation of noncyclic products; overall yields of CD are usually in the range of 35–50%. Of the three CDs formed (cyclomaltohexaose [α -CD], cyclomaltoheptaose [β -CD], and cyclomaltooctaose [γ -CD]), γ -CD is favored the least; its yields are generally 4–7%, based on total glucose-unit content of the substrate. The relative proportions of α -CD and β -CD can vary appreciably according to reaction conditions (such as time, temperature, and substrate concentration); however, in most instances, when a near-equilibrium state has been attained, the yield of β -CD is slightly higher than that of α -CD. Where insufficient time is allowed for attainment of this state, the yield of α -CD often exceeds that of β -CD. CGTase from *B. macerans* initially favors α -CD production; only in later stages of the reaction does the yield of β -CD approximate or exceed that of its α -homolog.

*Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. All programs and services of the US Department of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap.

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Although the precise mechanism for cyclization is not known, the equilibria shown in **Reactions 1 and 2** are believed to be involved **(1)**. The symbols G_n , G_m , and G_x represent



straight-chain maltooligosaccharides containing n , m , and x units of (1→4)- α -D-glucopyranose; cG_x is a cyclodextrin composed of x units. A glucose unit of the nonreducing end of a (1→4)- α -D-glucopyranosyl chain appears to be transferred by way of either O-4 or C-4 to either C-1 or O-1 of another glucose unit that functions as a suitable donor. α -, β -, and γ -CD are composed of 6, 7, and 8 glucose units, respectively. Their respective cavity diameters are approx 5, 7, and 8 Å, and their respective cavity volumes are 176, 346, and 510 Å³. Crystalline CDs exist as hydrates. Degree of hydration of α - and β -CDs varies only slightly over the relative humidity (RH) range of 24–70% at 25°C. Degree of hydration of γ -CD is moderately stable over the range of 24–50%; however, at 70% RH, it is almost twice that at 50% RH.

Because of the ability of CGTase to degrade CDs, presumably through coupling reactions between CDs and reaction mixture components, such as D-glucose and maltooligosaccharides, excessively prolonged reaction times can be detrimental to yield. Consequently, it is important that in many preparations of CDs, reaction time be carefully governed in order to achieve the best yield of either a particular homolog or a mixture of CDs. Determining when to terminate a reaction can occasionally necessitate periodic removal of small aliquot samples of the reaction mixture and subsequently analyzing them for individual CDs by high-performance liquid chromatography (HPLC).

Product ratios and total CD yields are greatly influenced by the formation of either highly insoluble or highly stable inclusion compounds in the reaction mixtures. CD inclusion compounds are the complexes formed by the entrapment of a guest molecule (complexant) within the cavity of a CD molecule (the host). Enhancement of α -CD production is best accomplished through use of the straight-chain alkanols nonan-1-ol, decan-1-ol, or dodecan-1-ol as guest compounds **(2,3)**. Straight-chain alkanols of molecular weight lower than that of nonan-1-ol favor β -CD production; those of molecular weight higher than that of dodecan-1-ol lead to lower α -CD yields accompanied by substantial amounts of β -CD. Branched alkanols favor only β -CD. The currently high cost of nonan-1-ol precludes its general use as a complexant. β -CD production is highly favored by a wide variety of guest compounds, among which are

aliphatic and aromatic hydrocarbons (2–4), certain branched alkanols (3), and cycloalkanones of ring size smaller than 12 atoms (5). The production of γ -CD in good yield can be accomplished most practically by the use of certain cyclic complexants having 12 or more ring atoms (5–8).

Starch suitable for producing CDs can be obtained from numerous plant sources, such as corn, rice, wheat, oats, potatoes, and tapioca. It should be clean and free of lipids and proteinaceous material, both of which might conceivably diminish either the ability of CGTase to effect starch conversion or the ease of isolating CDs subsequent to conversion. Starches composed largely of amylopectin make the best substrates (9). They are easily gelatinized and liquefied—a prerequisite for effective starch conversion.

For this chapter, pure corn amylopectin was chosen as substrate for all conversion reactions. Decan-1-ol was selected as complexing agent to enhance α -CD production; however, care must be exercised because of its toxicity. For β -CD productions, β -specific complexants of several different types (cyclohexane, cyclooctane, cyclododecene, isooctane, and *t*-butanol) were selected to illustrate the diversity of applicable guest compounds. Aromatic and halogenated hydrocarbons were excluded from consideration because of their high toxicities. Cyclododecanone and cyclotridecanone were chosen for enhancing γ -CD production (see **Note 1**); both can be easily recovered for subsequent reuse.

Debranching enzymes (e.g., pullulanase and isoamylase) in combination with *B. macerans* CGTase and a complexant that is selective for either α -, β -, or γ -CD can convert starch or maltodextrin into CDs in yields generally higher than those obtained in the absence of debranching enzyme (9). The methodologies described in this chapter include procedures for conducting reactions both with and without debranching enzyme. The use of amyloglucosidase to eliminate starch fragments (maltooligosaccharides and maltodextrins) from reaction-product mixtures and thus ensure greater purity of the isolated CDs is not essential and will not be employed. All CDs prepared by means of the procedures described herein are essentially free of such contaminants. Typical yields and results of HPLC analyses performed on isolated products will be provided.

Table 1 summarizes some of the physical properties of CDs. Because water of hydration can vary with RH, CDs should be stored in an environment of constant relative humidity.

2—

Materials

1. HPLC apparatus: a combination of a solvent delivery system, a DuPont Zorbax NH₂ column (4.6 × 250 mm), a column heater, a differential refractometer (detector), and a computing integrator; volume of the sample loop, 20 μ L. Zorbax columns are available through MAC-MOD Analytical, Inc. (Chadds Ford, PA).

Table 1
Physical Properties of Cyclodextrins

CD	Molecular weight	mp, °C	Water solubility, g/mL				Specific rotation (°) [α] ²⁵ _D
			3°C	25°C	45°C	60°C	
α	972.86	278 (decomposes)	5.8	12.8 ^a	2.90 ^a	66.2 ^a	150.5 ± 0.5 ^b
β	1135.01	260 (decomposes)	0.8	1.8 ^a	4.5 ^a	9.0 ^a	162.5 ± 0.5 ^b
γ	1297.15	267 (decomposes)	10.1	25.6 ^a	58.5 ^a	129.2 ^a	177.4 ± 0.5 ^b

^aFrom data of Cerestar USA Inc., Hammond, IN.

^bFrom ref. **(10)**. Measurements were made on aqueous solutions (lg of anhydrous CD/100mL) at 25°C at the wavelength

^cEstimated from molecular models.

^dThis value is for a commercial sample of α-CD and is somewhat lower than the % H₂O of hydration for α-CD isolated chapter.

2. Distilling apparatus for azeotropic distillation: a combination of a Liebig condenser (jacket length, 300 mm; standard-taper 24/40 joints), a distilling head (with standard-taper 24/40 joint at the bottom and on side arm), a 500-mL round-bottom flask (with single standard-taper 24/40 neck) to serve as boiler, and a heating mantle controlled by a variable autotransformer. Around the lower end of the condenser is placed loosely a large wide-mouth receiver flask, which may, if desired, be cooled by an ice bath in order to recover high-melting complexants that must be collected without benefit of a water-cooled condenser. **No lubricants are used in sealing joints**, because of the possibility that such lubricants might contaminate the reaction mixture and form complexes with CDs. All preparative and analytical operations should be conducted with scrupulously clean glassware.

3. CD glucanotransferase (EC 2.4.1.19) from *B. macerans*: commercially available as an aqueous solution (Amano International Enzyme Co., Inc., Troy, VA) with an activity of approx 600 U/mL according to the method of Tilden and Hudson (**11**). The action of a 5- μ L portion of this CGTase solution (~ 3 U of activity) on a maltodextrin substrate at pH 6.0 and 60°C yields 16 mg of combined CDs in 30 min. CGTase is very stable at pH 7.0 when stored at 3°C; no significant change in activity occurs over a period of a year. Stability is very high at and above pH 7.0, and decreases rapidly below pH 6.0. Activity is highest between pH 5.0 and 7.0, and decreases rapidly outside this range. Thermostability is high at 25°C (CGTase at pH 7.0 remains active for at least one month at 25°C) but low at 60°C (at pH 7.0 the enzyme becomes virtually inactive after 12 h).

4. Amyloglucosidase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3 from *Aspergillus niger*; 37 U/mg of solid or 42 U/mg of protein; 1 U liberates 1.0 mg of D-glucose from starch in 3 min at pH 4.5 and 55°C). A stock solution of this enzyme, prepared by dissolving 1.6 mg of solid enzyme in 3.0 mL of deionized water, is stable for several months when stored in a freezer.

5. Pullulanase (amylopectin 6-gluconohydrolase; EC 3.2.1.41; from *Enterobacter aerogenes*): suspension in $(\text{NH}_4)_2\text{SO}_4$ solution; 45 U/mL; 1 U liberates 1.0 mol of maltotriose from pullulan/min at pH 5.0 and 25°C. Should the pullulanase be available only in solid form, a measured amount of the solid (calculated to provide the desired number of units for a particular reaction mixture) is dissolved in a few milliliters of water shortly before application of the enzyme.

6. Tris (stock buffer; 0.8 M): a solution of *tris*(hydroxymethyl)aminomethane hydrochloride (3.15 g) in water (20 mL) is treated dropwise with 1 M NaOH (approx 1.45 mL) to adjust the pH to 7.2. Addition of 2.6 mL of this solution to 100 mL of reaction mixture will give a Tris concentration of 0.02 M.

7. Streptomycin sulfate (stock solution for use as bactericide): Streptomycin sulfate (35 mg) is dissolved in water (1 mL). Refrigerate. Each 0.1 mL will impart to 100 mL of reaction mixture a concentration of 35 μ g/mL.

8. Acetonitrile-water (13:7, v/v) for HPLC: Prepared by mixing HPLC-grade acetonitrile (1857 mL) and water (1 L). Because of the toxicity of the organic solvent,

mixing should be conducted in a well-ventilated hood, and care must be taken to avoid contact with the skin and inhaling the vapors.

9. Corn amylopectin (11.5% water of hydration at 31% RH). A very pure grade of this starch is available from Sigma Chemical Co. (St. Louis, MO).

10. Decan-1-ol (decyl alcohol), >99%; bp 231°C; mp 7°C; toxic irritant.

11. *t*-Butanol (2-methyl-2-propanol), >99.5%; bp 117.7°C; flammable and toxic.

12. Cyclododecene (mixture of *cis* and *trans*), ~95%; bp 232–245°C; possibly harmful by inhalation or skin absorption.

13. Cyclohexane, >99.9%; bp 80.7°C; mp 6.5°C; flammable irritant; avoid contact with skin and breathing the vapor.

14. Cyclooctane, >99%; bp 151°C/740 mm; mp 10–13°C; flammable liquid; possibly harmful by inhalation or skin absorption.

15. Isooctane (2,2,4-trimethylpentane), 99.8%; bp 98–99°C; mp -107°C; flammable irritant.

16. Cyclododecanone, >99%; bp 85°C/1 mm; mp 59–61°C; no toxicological data available.

17. Cyclotridecanone, 97%; bp 146°C/11 mm; mp 30–31°C; no toxicological data available.

18. Diethyl ether, 99.8%; bp 34.6°C; mp -116°C; flammable and toxic.

19. Activated charcoal (Darco G-60).

3—

Methods.

3.1—

Analytical

1. CDs are separated by HPLC on a DuPont Zorbax NH₂ column (4.6 × 250 mm) at 40°C with acetonitrile-water (13:7) at 1.0 mL/min and detected by refractometry. CD reference standard for comparison with elution peaks is a mixture of α -, β -, and γ -CD (1–2 mg of each anhydrous CD/mL of aqueous solution).

2. All solutions are filtered by syringe through Millipore HV filter units of 0.45- μ m pore size (Millipore Corp., Bedford, MA) before injection.

3. Prior to HPLC analysis for CDs, reaction mixture or CD solutions containing maltooligosaccharides are treated with amyloglucosidase at pH 4.5 and 55°C for 1 h to eliminate maltooligosaccharides that would interfere with CD determinations. Generally, a 10- μ L portion of stock amyloglucosidase solution is sufficient to accomplish this removal in 4 mL of reaction mixture that has been diluted 30-fold. Such treatment degrades large malto-oligosaccharides almost entirely to D-glucose; minor, noninterfering amounts of maltose and maltotriose occasionally survive the degradative action of the enzyme (see **Note 2**). Enzyme inactivation prior to HPLC analysis is not necessary, provided chromatographic injection is carried out within several hours after the enzyme treatment. Analyses may be postponed indefinitely, without enzyme inactivation, if the solutions are frozen for safekeeping.

4. Calculation of CD yields is based on the total of D-glucose residues (in mmol) in the starch substrate. Each gram of anhydrous starch contains 6.167 mmol of D-glucose residues.

3.2—***Gelatinization of Starch***

1. A 10% (w/w) starch gel is prepared as follows. A mixture of water (70 mL) and starch (7.5 g, anhydrous basis) in a 250-mL Erlenmeyer flask is stirred at room temperature until homogeneous (no lumps).
2. The lower end of the flask is then immersed in a vessel of boiling water, and the contents are agitated by a rotary motion of the flask until the starch gelatinizes (about 2 min).
3. The flask is allowed to remain in the hot bath for a total of 15 min before removal and cooling to 25°C.

3.3—***Enzyme Inactivation***

The contents of a reaction flask are poured portionwise into a 250-mL Erlenmeyer flask containing 50 mL of rapidly boiling water. Rate of the addition is slow enough to prevent the temperature from dropping below 90°C. The emptied flask is rinsed with small amounts of water, and the rinsings are added to the boiling mixture. The temperature of the mixture is maintained at 100°C for 1 h by placing the flask (loosely stoppered) in a large beaker of boiling water.

3.4—***Removal of Complexant from Reaction Mixtures by Azeotropic Distillation***

After enzyme inactivation, the isolated complex (or the entire reaction mixture, if isolation of the complex is not practical) is placed in the 500-mL boiler flask of the distillation apparatus described in **Subheading 2**. Sufficient water is then added to the contents to make a total volume of about 400 mL. A boiling chip is introduced, and the mixture is brought to a rapid boil. Several times during the course of distillation, additional water must be added and the process continued until a total of 1 L of distillate has been collected. The volume of residual liquid should be about 100 mL at the time the operation is ended.

Azeotropic treatment of an aliquot sample (usually 0.5 mL) of reaction mixture is best accomplished in a screw-capped culture tube: After inactivation of the enzyme in a steam bath, the mixture is diluted with water and heated in a water bath at ~95°C, while N₂ is introduced at a controlled, moderate rate beneath the liquid surface by means of a capillary tube. The length of the operation (usually 2–3 h) varies according to the stability of the complex and the volatility of the complexant. Certain high-boiling complexants (such as decan-1-ol) are difficult to remove by this technique, unless the reaction mixture is first extracted with several portions of diethyl ether to remove the greater part of the complexant.

3.5—***Determination of Water of Hydration***

Weight loss that results from heating a weighed sample (~0.2 g) of CD or starch for 2 h under vacuum at 115°C in an Abderhalden is used to calculate % water of hydration.

3.6—**Conversion of 10% Solution of Amylopectin to α -CD****3.6.1—****With Decan-1-ol at 25° C in the Absence of Pullulanase**

1. In a 250-mL Erlenmeyer flask (with standard-taper 24/40 joint), gelatinize 8.48 g of amylopectin (11.5% H₂O; 7.50 g, anhydrous) in 70 mL of water (see Note [3](#)). **Cool to 25°C.**
2. Liquefy by adding CGTase (0.35 mL; 210 U) and stirring magnetically for 30 min; pH is ~5.5 (see Note [4](#)). Add stock streptomycin solution (0.1 mL).
3. Decan-1-ol (10 mL; 8.3 g) is introduced with stirring.
4. pH is adjusted to 7.0 with 0.1 M NaOH, the flask is stoppered, and rapid stirring is commenced. pH should be checked daily (see Note [5](#)).
5. At the end of 5 d, a second 0.35-mL portion of CGTase is added. Stirring is continued for another 5 d (see Note [6](#)).
6. Inactivate. Cool to 25°C, and stir rapidly overnight to reform the solid complex.
7. Cool to 10°C; then centrifuge at 11,000*g* for 30 min; decant; transfer solids to a boiler flask with approx 200 mL of water; add a stir bar and, under a well-ventilated hood, extract the stirred mixture three times with 200-mL portions of diethyl ether; boil off the residual ether on a steam pot, and conduct an azeotropic distillation to remove all remaining complexant.
8. Transfer contents of boiler flask to a 250-mL Erlenmeyer flask; add a stir bar, boiling chip, and 2 g of activated charcoal. Apply heat and boil for 10 min with stirring. Remove heat and allow to cool slowly to room temperature with continued stirring. Filter with suction in a medium-porosity 150-mL fritted-glass funnel, and wash the charcoal with several 10-mL portions of water.
9. Transfer the clear, colorless filtrate and washings to a 500-mL single-neck roundbottom flask (with standard-taper 24/40 joint) and evaporate thoroughly to constant weight on a rotary evaporator at 45°C. Yield, 4.8 g; 11.6% H₂O (RH 31%); HPLC analysis of anhydrous sample: 95.6% α 0% β 0% γ ~3% unidentified component (probably a branched CD).
10. Recrystallization from water at 3°C: In a small weighed beaker dissolve 4.6 g of α -CD from **step 9** in 15 mL of water on a steam pot. Continue heating until the weight of the contents is reduced to 9.0 g. Cover mouth of beaker with Parafilm or aluminum foil, and chill overnight at 3° C. Filter with suction in a mediumporosity fritted-glass funnel at 3°C, and wash with two 3-mL portions of cold (0°C) water. Yield: 2.9 g; 10.7% H₂O (31% RH). HPLC analysis of anhydrous sample: 98.2% α 0% β 0% γ .

3.6.2—**With Decan-1-ol at 25° C in the Presence of Pullulanase**

1. Gelatinize 8.48 g of amylopectin (11.5% H₂O) as in **Subheading 3.6.1., step 1.**
2. Liquefy with pullulanase (0.75 mL of aqueous NH₄SO₄ suspension; 34 U) by stirring magnetically for 4 h at 25°C; pH is ~5.5 (see Note [4](#)) **Add stock streptomycin solution (0.1 mL).**

3. Add CGTase (0.35 mL; 210 U) and decan-1-ol (10 mL; 8.3 g). Adjust pH to 7.0, and commence rapid stirring. Check the pH daily (see Note [5](#)).

4. Follow steps **Subheading 3.6.1., steps 5–9**. Yield: 6.5 g; 11% H₂O (31% RH). HPLC analysis of anhydrous sample: 100% α 0% β 0% γ

3.7—

Conversion of 10% Solution of Amylopectin to β -CD.

3.7.1—

With Cyclohexane, Cyclooctane, Cyclododecene, and *t*-Butanol at 25° C in the Absence of Pullulanase

1. In a 250-mL Erlenmeyer flask (with standard-taper 24/40 joint), gelatinize 8.48 g of amylopectin (11.5% H₂O; 7.50 g, anhyd.) in 70 mL of water. Cool to 25°C.
2. Liquefy by adding CGTase (0.40 mL; 240 U) and stirring magnetically for 30 min; pH is ~5.5 (see **Note 4**). Add stock streptomycin solution (0.1 mL).
3. Introduce complexant (20 mL of either cyclohexane, cyclooctane, isooctane, cyclododecene, or *t*-butanol) with stirring, adjust pH to 7.0 with 0.1 M NaOH, and stir rapidly for 5 d.
4. Inactivate the enzyme at 100°C, and cool to room temperature. Introduce 10-mL portions of complexant to replace any that might have been lost through evaporation during enzyme inactivation.
5. Except where *t*-butanol was used as a complexant, all mixtures must be stirred at 25°C, preferably overnight, to allow time for reformation and reprecipitation of complex (see **Note 7**). For reactions involving *t*-butanol, transfer entire reaction mixture to a boiler flask, dilute with water to ~400 mL and skip **step 6** (see **Note 8**).
6. Cool to 15°C and centrifuge at 11,000*g* for 30 min; decant; transfer solids to a boiler flask with ~400 mL of water.
7. Azeotropic distillation.
8. Transfer contents of boiler flask to a 250-mL Erlenmeyer flask; add a stir bar, boiling chip, and 2 g of activated charcoal. Boil for 10 min with stirring and then allow to cool slowly to 60°C with continued stirring (see **Note 9**). Immediately filter with suction in a 150-mL fritted-glass funnel. (**Caution:** Because there may be a tendency for the warm filtrate to boil or bump under these conditions, the size of the filter flask should be sufficiently large to prevent loss of filtrate through sudden boiling.) Wash the charcoal with several 10-mL portions of water (60°C), and transfer the combined filtrate and washings to a 500-mL single-neck, roundbottom flask (with standard-taper 24/40 joint) for evaporation to dryness at 45°C in a rotary evaporator.
9. With the aid of water (40–50 mL) from a wash bottle, scrape the crude β -CD from the flask walls, and transfer entirely to a 100-mL beaker. Heat on a steam pot to dissolve, and then evaporate to an appropriate volume (where there is approx 2 g of water for each g of solid). Let stand overnight at 25°C to crystallize.
10. Filter with suction in a small (30-mL), medium-porosity, fritted-glass filter funnel. Wash with 5 mL of water. Allow to dry in open air, and then equilibrate at 31% RH.

Table 2 presents results of conversion reactions conducted in the absence of a debranching enzyme.

Table 2
Data on β -CD Produced at 25°C in the Absence of Pullulanase

Complexant	Yield of β -CD, g	Water of hydration, %	% α	HPLC analysis ^a		
				% β	% γ	% Other
Cyclohexane	4.9	13.2	0	100.8	0	0
Cyclooctane	4.8	13.3	0	100.9	0	0
Cyclododecene	4.6	13.5	0	100.0	0	0
Isooctane	4.8	13.3	0	100.0	0	0
<i>t</i> -Butanol	4.5	12.4	0	98.0	0	~2 ^b

^aAnhydrous sample.

^bUnidentified component with retention time slightly greater than that of α -CD; probably a branched CD.

Table 3
Data on β -CD Produced at 25°C in the Presence of Pullulanase

Complexant	Yield of β -CD, g	Water of hydration, %	% α	HPLC analysis ^a		
				% β	% γ	% Other
Cyclohexane	5.2	13.5	0	100.2	0	0
<i>t</i> -Butanol	6.2	13.3	0	99.7	0	0

^aAnhydrous sample.

3.7.2—

With Cyclohexane and *t*-Butanol at 25° C in the Presence of Pullulanase

1. Same as **Subheading 3.7.1., step 1.**
2. Liquefy gel at 25°C by adding CGTase (1.2 mL; 720 U) and stirring magnetically for 15 min; pH ~5.5 (see **Note 4**). Add stock streptomycin solution (0.1 mL).
3. Add pullulanase (0.75 mL of aqueous NH₄SO₄ suspension; 34 U).
4. Introduce complexant (20 mL) with stirring; adjust pH to 7.0 with 0.1 M NaOH; stir for 6 d.
5. Same as **Subheading 3.7.1., steps 4–10.**

Table 3 presents results of conversion reactions conducted in the presence of a debranching enzyme.

3.8—

Conversion of 10% Solution of Amylopectin to γ -CD

3.8.1—

With Cyclododecanone and Pullulanase at 60° C

1. In a 250-mL Erlenmeyer flask (with standard 24/40 joint), gelatinize 8.48 g of amylopectin (11.5 H₂O; 7.50 g, anhydrous) in 70 mL of water. Cool to 25°C.

2. Liquefy by adding pullulanase (32 U) and stirring magnetically for 6 h at 25°C. pH is 5.3 (see **Note 4** and **12**). Addition of streptomycin is not essential.
3. Add Tris (5 mL of stock solution) and first increment of CGTase (0.35 mL; 210 U) (see **Note 10**). Stir for 45 min; then add cyclododecanone (3.5 g). Warm to 61°C (no higher) to melt the complexant and effect its even distribution. Adjust the pH to 7.0 with 1 M NaOH, and place stoppered flask in a shaker cabinet maintained at 60°C. Agitate vigorously (see **Note 11**).
4. Apply additional increments of CGTase (0.35 mL each) twice daily for a total of 12 increments, with at least an 8-h interval between additions.
5. Check progress of reaction by subjecting a 0.5-mL aliquot of stirred reaction mixture to HPLC analysis to determine the ratio of γ -CD to β -CD. If the ratio is 20:1 or greater, consider the reaction complete. If not, continue applying increments of CGTase until the desired ratio is achieved.
6. Eliminate any remaining enzyme activity by placing the flask (loosely stoppered) in a beaker of boiling water for 1 h. Cool to 25°C, and stir rapidly overnight to reform the solid complex. Then stir for 1 h in an ice bath, and filter with suction in a medium-porosity, 150-mL, fritted-glass filter funnel. Wash the solids several times with 20-mL portions of cold (0°C) water.
7. Transfer completely the solids to a 500-mL boiler flask, and subject to azeotropic distillation. Cooling of the condenser with water is not recommended because of the high melting point of the complexant.
8. Transfer the remaining liquid (~100 mL) to a 250-mL Erlenmeyer flask, add 2 g of activated charcoal, boil for 10 min, and allow to cool slowly to room temperature.
9. Filter with suction in a medium-porosity, fritted-glass funnel, and wash with several 10-mL portions of water. Transfer the combined filtrate and washings to a 500-mL, round-bottom flask (standard-taper 24/40 joint), and evaporate to constant weight at 45°C in a rotary evaporator. Yield, 4.4 g; 8.5% H₂O; HPLC analysis of anhydrous sample: 0% α ~1% β ; 99.0% γ .
10. Recrystallization from water at 3°C. Into a weighed beaker place 4.2 g of slightly impure γ -CD isolated in **step 9**. Dissolve in 15 mL of water on a steam pot, and evaporate until the weight of the contents is reduced to 8.2 g. Cover the beaker mouth with Parafilm or aluminum foil, and place in a refrigerator at 3°C overnight. At 3°C filter with suction in a small fritted-glass funnel (medium porosity). Wash with two 3-mL portions of cold (0°C) water. Yield, 3.2 g (at 31% RH); 8.9% H₂O. HPLC analysis of anhydrous sample: 0% α 0% β ; 100% γ ; 0% other.

3.8.2—

With Cyclotridecanone and Pullulanase at 40° C

1. Same as **Subheading 3.8.1., step 1**.
2. Liquefy by adding CGTase (1.05 mL; 630 U) and stirring magnetically for 20 min at 25°C. pH is ~5.5 (see **Note 4**). Addition of streptomycin is not essential.
3. Add Tris (5 mL of stock solution), pullulanase (32 U), and cyclotridecanone (2.3 g; 2.5 mmol). Adjust pH to 7.0, and place stoppered flask in a shaker cabinet at 40°C (see **Note 11**).

4. At the end of 3 d, add a second portion of CGTase (1.05 mL). Readjust pH to 7.0, if necessary, and continue shaking at 40°C for two more days.
5. Same as **Subheading 3.8.1., steps 5–9**. Yield, 5.2 g (at 31% RH); 10.5% H₂O. HPLC analysis of anhydrous sample: 0% α ; ~2% β ; 97.4% γ .
6. Recrystallization from water of 4.2 g of impure γ -CD isolated in **step 5** follows the same procedure as that in **Subheading 3.8.1., step 10**. Yield, 3.1 g; 8.8% H₂O. HPLC analysis of anhydrous, sample: 0% α ; 0% β ; 100% γ ; 0% other.

4—

Notes

1. The use of cyclododecanone as complexant to enhance γ -CD production, unlike the use of cycloalkanones of larger ring size, necessitates applying CGTase in small increments over a prolonged period of time. A single increment, regardless of size, favors the production of β -CD (5).
2. Should reaction mixtures or solutions of CDs be treated with amyloglucosidase to eliminate any noncyclic maltooligosaccharide and maltodextrin components, consideration must be given to the possibly significant degradative action of this enzyme on γ -CD. α -CD and β -CD are relatively inert. To illustrate, when 4 mL of 1.25 mM γ -CD (~40 mmol of glucose residues) are treated with amyloglucosidase (10 μ L of stock solution; 0.2 U of activity) at pH 4.5 and 55°C for 1 h, 2.5% of the γ -CD is lost through conversion to D-glucose (1.0 mmol). A 5% loss occurs after 2 h; D-glucose and a smaller amount maltose are products. (A single HPLC operation permits the quantitative determination of γ -CD, D-glucose, and maltose). Thus, when use is made of amyloglucosidase to determine the purity of a γ -CD sample, an appropriate correction factor must be applied to take into account the loss from degradation.

Amyloglucosidase that has been applied to a reaction mixture can be effectively and completely inactivated at pH 7.0 without significant action on γ -CD by very rapidly heating the mixture to 100°C and keeping it at this temperature for 30 min.
3. In the preparation of α -CD, but not that of either β -CD or γ -CD, initial starch concentrations of <10% (w/w) should not be used because of an adverse effect that lower starch concentrations can have on yields (unpublished observations of the author).
4. The flask containing the gel and liquefying enzyme must first be agitated manually in order to weaken the gel structure sufficiently to permit rotation of the stir bar.
5. The pH should not be allowed to fall below 6.0, because below this level the enzyme stability might decrease more rapidly than is desirable. Adjustments of pH are made by additions of NaOH or HCl.
6. Approximately the same yield of α -CD can be obtained by using a single 70-mL application of CGTase (420 U) and a 6-d reaction period.
7. Elevated temperatures can cause extensive dissociation and dissolution of CD complexes in aqueous media.
8. The *t*-butanol- β -CD complex is highly soluble at room temperature and is not effectively isolated by precipitation.

9. A temperature lower than 60°C for the filtration operation is not recommended, because of the relatively low solubility of β -CD at or near 25°C.

10. Use of Tris buffer eliminates the need for frequent pH checks. However, as a precautionary measure, it is recommended that a pH determination be made every 2 or 3 d during the course of a reaction. Reduction of the temperature is not essential for the purpose of such measurements.

11. Shaking should be in a rotary fashion and at a rate (~200 rpm) fast enough for efficient mixing, but not so fast that the mixture splashes into the neck of the flask. Use of thin Teflon tape to seal glass-stoppered joints can eliminate the possibility of stoppers becoming difficult or impossible to remove because of the cementing ability that certain reaction-mixture components have once they enter the joint.

12. Because of the relatively low thermostability of pullulanase at 60°C, liquefaction of starch gel (through debranching) is first effected with pullulanase alone at 25°C prior to adding complexant and CGTase and raising the temperature to 60°C, the desired level for conversion. Conversions of starch into γ -CD in the presence of the complexant cyclododecanone are best conducted at or very close to 60°C. Use of lower temperatures can result in much lower yields; higher temperatures necessitate larger amounts of CGTase because of lower thermostability of the enzyme.

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9—

Production of Microbial Glycolipids

Siegmund Lang

1—

Introduction.

Some microorganisms are able to convert preferably carbohydrates, *n*-alkanes (C10 to C20), and triglycerides (fatty acids of C10 to C22) into glycolipids. The carbon sources may be used separately or in combination with each other. As for biosynthetic pathways, these include both degradation and new synthesis of sugar lipids, as well as direct incorporation, elongation, or modification of precursor molecules. Reviews recently published are those of Desai and Desai (**1**) and Lang and Wagner (**2,3**), indicating that in general the following patterns of glycolipid production by microbial cultivation are possible:

1. Growth-associated production.
2. Production under growth-limiting conditions.
3. Production by resting cells (free or immobilized).

In most cases, the yields of method 1 are substantially lower than those of methods 2 and 3. To allow glycolipid overproduction for a long time, active enzymes (provided during the preceding exponential growth phase), sufficient amounts of carbon sources, as well as a high-energy potential are necessary. The final products isolated by precipitation, solvent extraction, or crystallization are not single glycolipids, but mixtures of similar compounds, differing in the number of carbohydrate and fatty acid/fatty alcohol units as well as chain length of lipid moieties. However, the fundamental type of glycolipid from a special microorganism may not be changed by variation of cultivation parameters.

In the following, the microbial producers—yeasts and bacteria—and molecular structures of glycolipids have to be presented. In most cases, the microorganisms are available from culture collections of US, The Netherlands, or of Germany.

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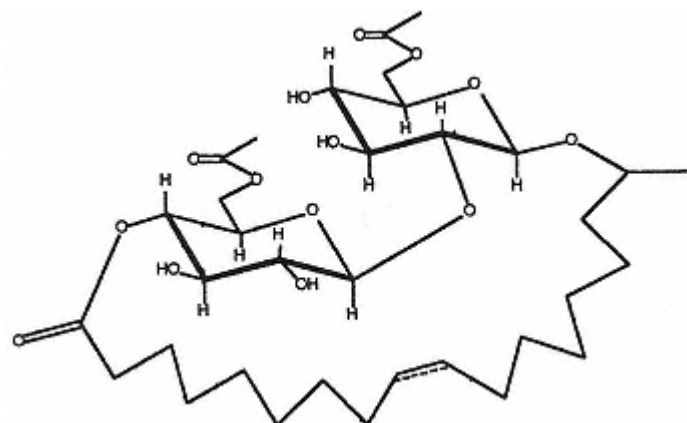


Fig. 1.
Lactonic sophorose lipid from *C. bombicola* (major component).

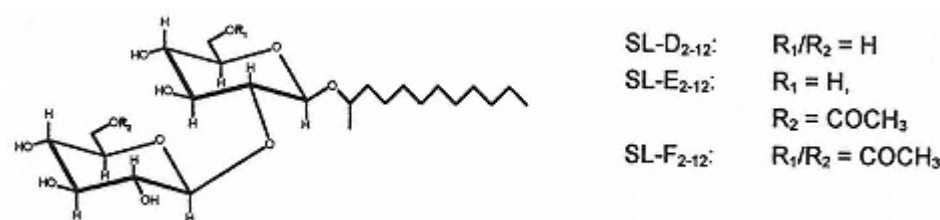


Fig. 2.
Dodecyl-sophorosides from *C. bombicola* (major components).

1.1—

Lactonic Sophorose Lipids from Candida bombicola

Beginning with the best-developed process until now, instead of shake-flask experiments, it is possible to describe immediately a bioreactor cultivation. The yeast *C. (formerly Torulopsis) bombicola* ATCC 22214 is able to convert blends of carbohydrate/lipophilic compounds into sophorose lipids. This biosurfactant contains the disaccharide sophorose and, by glycosidic and lactonic linkages, a 17-hydroxy C18 (un)saturated fatty acid (see **Fig. 1**). As suitable precursors, glucose/rape seed oil ethyl esters (**4**), glucose/canola oil (**5**), and glucose/oleic acid (**6**) have been used. The yields were in the range of 0.60–0.70/g carbon sources.

1.2—

Alkyl-Sophorosides from C. bombicola

Recently a novel type of sophorose lipids produced by the yeast mentioned above was detected (**Fig. 2**). Using blends of glucose/2-alkanols (C12 to C16),

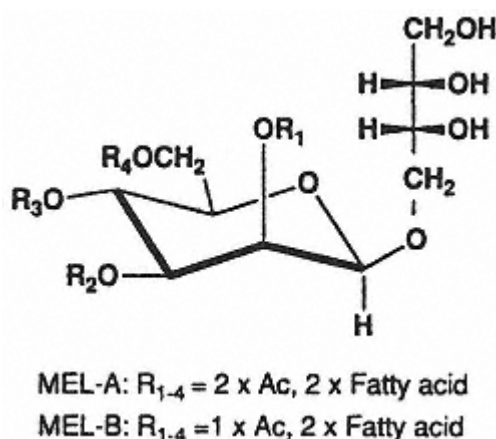


Fig. 3.
Mannosylerythritol lipids from *C. antarctica*
(major components).

a direct attachment of the secondary alcohol to the disaccharide was observed (7). According to the early stage of studies on this process, shake-flask experiments will be presented.

1.3—

Mannosylerythritol Lipids from *C. antarctica*

Using yeast species, such as *Candida* sp. B-7 and *Candida antarctica* T-34, a Japanese group was successful in producing mannosylerythritol lipids (MEL) from *n*-alkanes or vegetable oils (8,9). These strains not available at present, but the authors also examined the MEL productivity of three stock strains of *C. antarctica*. Of these, strain T-34 was the best producer, but similar yields were obtained with *C. antarctica* CBS 6821 (=ATCC 32657, DSM 70725): 27 g/L from 80 mL/L of soybean oil. The relative amounts of the individual MELs were found to vary slightly among the strains. However, with all strains, the major components were MEL-A and MEL-B shown in Fig. 3.

1.4—

Trehalose Lipids from *Rhodococcus erythropolis*

During cultivation on *n*-alkanes *R. erythropolis* DSM 43215 (patent strain; until now not available from the German culture collection) is able to synthesize a trehalose lipid mixture consisting of an anionic trehalose-2,2',3,4-tetraester (90%) and of trehalose-mono- and dicorynomycolates (10%); for molecular structures, see Fig. 4. Preconditions for an overproduction are nitrogen limitation, and temperature and pH shifts. The optimum carbon source according to our experience was technical-grade *n*-C10, which led to 0.35 g glycolipid/g *n*-alkane (10,11). However *n*-dodecane is also a good substrate. Recently, Espuny et al. (12) also produced trehalose lipids using *Rhodococcus* sp.

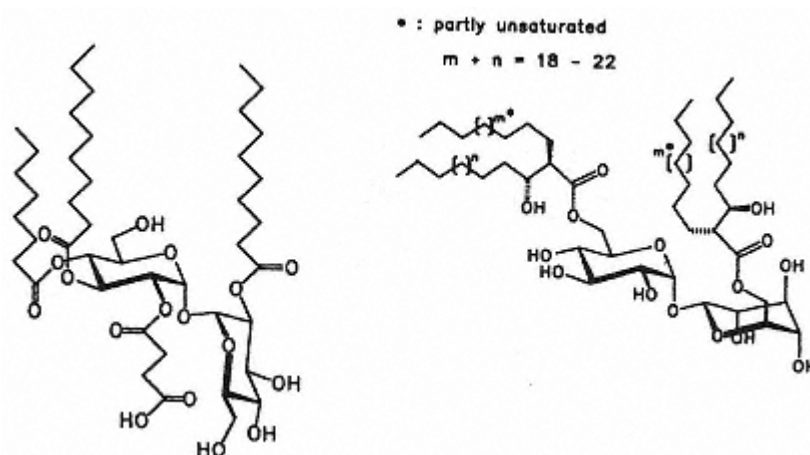


Fig. 4.
Trehalose-2,2',3,4-tetraester (left) and trehalose-dicorynomycolates (right) from
R. erythropolis (major components).

51T7. Slight modifications in molecular structure of trehalose lipids have been reported by Uchida et al. (13,14) from strain SD-74: 2,2',3,4-di-*O*-succinoyl-di-*O*-alkanoyl- α,α -trehalose and 2,3,4-mono-*O*-succinoyl-di-*O*-alkanoyl- α,α -trehalose.

1.5—

Rhamnose Lipids from *Pseudomonas* Species

Rhamnose lipids (Fig. 5) are excreted by *Pseudomonas* species when grown on glycerol, sorbitol, glucose, *n*-alkanes, or vegetable oils. Using *n*-C14, 15 (technical-grade, 80 g/L) our group obtained 13 g/L when culturing *Pseudomonas* sp. DSM 2874 for 7 d under *N*-limitation conditions (15). With resting cells in a buffer solution and on *n*-C14 (40 g/L) as carbon source, 10 g/L products, that means 0.25 g/g substrate, were isolated (16). Using *Pseudomonas aeruginosa* PG201 (=DSM 2659) and corn oil as carbon source Ochsner et al. derived 0.48 g/g substrate and a productivity of 2 g/L h; the plant has been based on a continuously stirred-tank reactor harboring an external loop (17). These bacteria are patent strains deposited at the German culture collection (DSM), and unfortunately not available at present.

The present level of overproduction and the use of rhamnolipids as biosurfactant or alternatively as a source for rhamnose both have great potential for an industrial application. However, the disadvantage is that special permission would be required to make use of *P. aeruginosa* as a production strain, because it is an opportunistic human pathogen and is therefore not G.R.A.S. (generally regarded as safe). For scientific experiments, taking into consideration the precautions for working procedures with risk class 2 microorganisms,

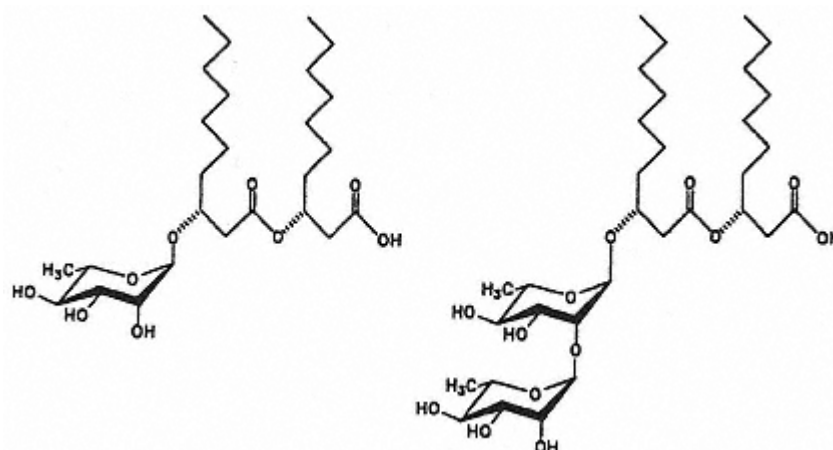


Fig. 5.
Rhamnolipids from *Pseudomonas* species.

the rhamnolipid production route using *Pseudomonas* sp. DSM 2874 in 100-mL cultures has been described (**Subheading 2.5.**).

1.6—

Emulsan (Lipoheteropolysaccharide) from *Acinetobacter calcoaceticus*

Emulsan is a lipoheteropolysaccharide bioemulsifier produced by *A. calcoaceticus* ATCC 31012 (formerly *Arthrobacter* RAG-1). Similarly to microbial rhamnolipid producers, this strain also belongs to risk class no. 2 (in Germany). In 1979, Rosenberg et al. (18) reported the isolation and partial purification of Emulsan after culturing the bacterium on a minimal medium, including *n*-hexadecane or ethanol as carbon sources. Later on, Shabtai and Wang (19) found that soybean oil or free fatty acids (oleic acid) increased the specific production of Emulsan (g/g carbon source) twofold.

Since there are disadvantages with downstream processing (separation of residual lipophilic substrates), an ethanol cultivation achieved in shake flasks (20) will be presented. The molecular composition depends on the carbon source and is cited for ethanol:

Monomers: galactosamine/glucose/galactose/unidentified substance 10/1/1/6

Fatty acid content: 12%

Protein content: 15%

Molecular weight: 10^6 Daltons

1.7—

Surface-Active Properties

The glycolipids mentioned above are able to lower the surface tension of water from 72 mN/m to values between 44 and 25 mN/m; see **Table 1**.

Table 1
Influence of Crude Glycolipids on the Surface Tension of Water (CMC Values: 10 to 200 mg/L) at Temperatures of 25–40°C

Glycolipids	Minimum surface tension, (mN/m)
Lactonic sophorose lipids	35
Dodecyl-sophorosides	30
Mannosylerythritol lipids	28
Trehalose lipids	26
Rhamnose lipids	25
Emulsan	44

2—

Materials

2.1—

Method 1: Microbial Production of Lactonic Sophorose Lipids.

1. Microorganism: *C. bombicola* ATCC 22214, available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20 852.
2. Medium for agar slants: yeast malt (YM) agar (Difco, Detroit, MI), consisting of 0.3% Bacto-yeast extract (YE), 0.3% Bacto-malt extract, 0.5% Bacto-peptone, 1% Bacto- dextrane, and 2% Bacto-agar, made up to 1 L with deionized water. Use immediately.
3. Media for seed cultures no. 1 and no. 2: 100 g/L glucose, 10 g/L yeast extract, 1 g/L urea. Use immediately.
4. Fermentation medium: *see* under **item 3**, with additional feed of glucose (0.6 g/L h) and of oleic acid (0.4 g/L h), starting after 36 h of cultivation. Use a 50-L bioreactor equipped with three six-bladed Rushton turbines.
5. Ethanol/*n*-butanol = 1/1 (v/v). Stable at room temperature.
6. 1 M KH_2PO_4 / K_2HPO_4 , pH 7.0. Stable at room temperature.
7. Glucose analyzer (glucose oxidase): Accutrend® alpha, Boehringer, Mannheim, GER.
8. Gas chromatograph, e.g., Model 438 A from Chrompack (Frankfurt, GER), equipped with a Chrompack CP-Sil 50 column connected to an FID.
9. Test kit Lato N, LCK 338 (Lange, Düsseldorf, GER).
10. Materials for thin-layer chromatography:
 - a. Silica gel 60 plates (no. 5553; Merck, Darmstadt, GER).
 - b. Solvent system: chloroform/methanol/water = 65/15/2 (v/v/v).
 - c. Detecting reagent: α -naphthol reagent: 10.5 mL α -naphthol solution (15% in ethanol), 6.5 mL sulfuric acid (conc.), 40.5 mL ethanol, and 4 mL distilled water.

d. Densitometer TLC scanner CD 60 (Desaga, Heidelberg, GER). Solutions are stable at room temperature.

11. Autotensiomat LMT 101 (Lauda/Wobser GmbH & Co. KG, Lauda-Königshofen, GER).

12. Medium-pressure liquid chromatography: Pump 681 (maximum 12 bar) and column, 460 × 49 mm (Büchi, Eisligen, GER), filled with silica gel 60, 70–230 mesh ASTM (Merck); chloroform/methanol mixtures (98/2 to 60/40; v/v) as developing system.

2.2—

Method 2: Microbial Production of Dodecyl-Sophorosides

1. See Subheading 2.1., item 1

2. See Subheading 2.1., item 2.

3. Medium for seed culture: 100 g/L glucose, 5 g/L sodium citrate · 3 H₂O, 1.5 g/L NH₄Cl, 1 g/L KH₂PO₄, 1 g/L yeast extract, 0.7 g/L MgSO₄ · 7 H₂O, 0.5 g/L NaCl, 0.27 g/L CaCl₂ · 2 H₂O, 0.16 g/L K₂HPO₄ · 3 H₂O and 1 L distilled water. Use immediately.

4. Fermentation medium: see under **item 3**; additionally three portions of 8 g/L 2-dodecanol during the cultivation time.

5. See Subheading 2.1., item 5.

6. See Subheading 2.1., items 6.

7. See Subheading 2.1., item 7.

8. See Subheading 2.1., item 8.

9. Merckoquant® 10024 ammonium test (Merck).

10. Thin-layer chromatography:

a. Silica RP-18 plates (Merck).

b. Solvent system: methanol/water = 80/20 or 90/10 (v/v).

c. Detecting reagents: α-Naphthol reagent: 10. 5 mL α-naphthol solution (15% in ethanol), 6.5 mL sulfuric acid (conc.), 40.5 mL ethanol, and 4 mL water (for glycolipids); vanillin reagent: 0.5 g vanillin in 100 mL sulfuric acid/ethanol (4/1, v/v) (for residual 2-dodecanol).

11. Autotensiomat LMT 101 (Lauda/Wobser GmbH & Co. KG).

2.3—

Method 3: Microbial Production of Mannosylerythritol Lipids

1. Microorganism: *C. antarctica* CBS 6821, available from the Centraalbureau voor Schimmelcultures, Julianalaan 67, 2628 BC Delft, The Netherlands.

2. Medium for agar slants: potato-dextrose agar (Difco). Use immediately.

3. Seed culture medium: 60 g/L glucose, 1 g/L NH₄NO₃, 0.5 g/L KH₂PO₄, 0.5 g/L MgSO₄ · 7 H₂O, 1 g/L yeast extract, and tap water. Use immediately.

4. Fermentation medium: 80 mL/L soybean oil, 2 g/L NaNO₃, 0.2 g/L KH₂PO₄, 0.2 g/L MgSO₄ · 7 H₂O, 1 g/L yeast extract, and tap water. Use immediately.

5. Materials for thin-layer chromatography: *see Subheading 2.1., item 10.* Densitometer: TLC scanner CS-920, Shimadzu or alternatively from another company.
6. Anthrone solution: 0.2% anthrone in 75% (v/v) sulfuric acid (**precaution!**). Stable at room temperature.
7. Column (3 × 40 cm) of silica gel (Wakogel C-200; Wako), equilibrated with chloroform (**precaution!**).
8. Autotensiomat LMT 101 (Lauda/wobser GmbH & Co. KG).

2.4—

Method 4: Microbial Production of Trehalose Lipids

1. Microorganism: *R. erythropolis* DSM 43215, a patent strain, not yet available from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany.
2. Medium for agar slants: 10.0 g/L *n*-dodecane, 1.5 g/L $(\text{NH}_4)_2\text{HPO}_4$, 0.5 g/L KH_2PO_4 , 1.0 g/L $\text{K}_2\text{HPO}_4 \cdot 3 \text{H}_2\text{O}$, 0.5 g/L $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 0.1 g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.1 g/L KCl, 1.0 g/L yeast extract, 0.5 mL trace metal stock solution (composition: $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ 11 g/L, $\text{CoSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.3 g/L, KJ 0.01 g/L, EDTA 5.0 g/L, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 6 g/L, $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ 1.0 g/L, $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ 0.04 g/L, H_3BO_3 0.06 g/L), 20.0 g/L agar (Difco); adjust to pH 7.0 with 10% NaOH solution. Use immediately.
3. Seed culture medium: *see* medium for agar slants, but omit agar.
4. Fermentation medium: 60.0 g/L *n*-C12, 1.0 g/L $(\text{NH}_4)_2\text{SO}_4$, 1.0 g/L yeast extract, 1.0 g/L H_3PO_4 (85%), 1.24 g/L citric acid $\cdot \text{H}_2\text{O}$, 0.2 g/L $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.02 g/L $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 1.1 g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.5 g/L $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 0.5 g/L KH_2PO_4 , 0.05 g/L $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$; adjust to pH 7.0 with 10% NaOH solution. Use immediately.
5. Ethanol/butan-1-ol/chloroform = 10/10/1 (v/v/v).
6. Merckoquant® 10024 ammonium test (Merck).
7. Ca-acetate solution (20%, w/v).
8. Gas chromatograph Model 428 (Packard, Frankfurt, GER), glass column / SE 30 Chromosorb W, 1 m.
9. Materials for thin-layer chromatography: *see Subheading 2.1., item 10.*
10. Autotensiomat LMT 101 (Lauda/Wobser GmbH & Co. KG).

2.5—

Method 5: Microbial Production of Rhamnose Lipids

1. Microorganism: *Pseudomonas* sp. DSM 2874, a patent strain, not yet available from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany.

Precaution: risk class 2. Use hood and automatic sterilizer; prevent aerosols; special precaution in downstream processing!

2. Medium for agar slants: 4.42 g/L $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$, 3.4 g/L KH_2PO_4 , 2.0 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.4 g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.4 g/L $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.4 g/L citric acid $\cdot 2 \text{H}_2\text{O}$, 0.04 g/L $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.005 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.0012 g/L NH_4 heptamolybdate $\cdot 4 \text{H}_2\text{O}$, 20 g/L glucose and 20 g/L agar (Difco); adjust pH to 6.8. Use immediately.
3. Seed culture medium: *see* medium for agar slants, but omit agar and replace glucose by 20 g/L *n*-tetradecane. Use immediately.

4. Fermentation medium: see seed culture medium, but take 60 g/L *n*-tetradecane. Use immediately.
5. Ethanol/butan-1-ol/chloroform = 10/10/1 (v/v/v).
6. Merckoquant® 10024 ammonium test (Merck).
7. Ca-acetate solution (20%, w/v).

8. Gas chromatograph Model 428 (Packard, Frankfurt, GER), glass column / SE 30 Chromosorb W, 1 m.

9. Materials for thin-layer chromatography: *see Subheading 2.1., item 10*; One exception: for developing, use chloroform/methanol/acetic acid = 65/15/2 (v/v/v).

2.6.—

Method 6: Microbial Production of Emulsan

1. Microorganism: *Acinetobacter calcoaceticus* ATCC 31012, available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. **Precaution:** risk class 2 (*see* working instructions **Subheading 2.5., item 1**).

2. Medium for agar slants: Nutrient agar (Difco). Use immediately.

3. Media for seed cultures nos. 1 and 2: 18.3 g/L $K_2HPO_4 \cdot 3 H_2O$, 6.0 g/L KH_2PO_4 , 4.0 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7 H_2O$, 1 L distilled water, 5 mL/L ethanol. Use immediately.

4. Fermentation medium: *see* medium for seed culture. Use immediately.

5. Merckoquant® 10024 ammonium test (Merck).

6. UV Test combination 176290 for ethanol (Boehringer Mannheim, GER).

7. Anthrone reagent: 1 L 72% sulfuric acid, 10 g thio urea, 0.5 g anthrone.

8. Visking® Dialysis Tubing 36/32 (27 mm), mol-wt exclusion limit: 12,000–14,000 (Serva Feinbiochemica GmbH, Heidelberg, GER).

3—

Methods

3.1—

Method 1: Microbial Production of Lactonic Sophorose Lipids

1. Sterilize all media at 121°C and 1 bar overpressure for 20 min.

2. For maintaining *C. bombicola* ATCC 22214 in your laboratory, incubate the yeast strain on agar slants at 27°C for 48 h, and deposit at 4°C.

3. Transfer a loopful of cells into a 500-mL Erlenmeyer shake flask (with two inserts) containing 100 mL of seed culture medium; inoculate five flasks in parallel. After culturing for 36 h at 27°C on a rotary shaker (100 rpm), transfer 50-mL portions into 10 500-mL cultures placed in 2-L Erlenmeyer shake flasks (two inserts). Cultivate as mentioned above. Then combine these seed cultures, and transfer to the 50-L bioreactor filled with 45 L fermentation medium. Work at 250 rpm, 27°C, and at an aeration rate of 0.6 vol air/vol culture suspension and min. Do not adjust the initial pH value (5.2) until it reaches pH 3.5; now keep it constant with 5 M NaOH. After 36 h of cultivation time, start an additional feed of glucose (0.6 g/L) and oleic acid (0.4 g/L); finish feeding after 180 h.

4. Check the time-course of cultivation by using the following analytical methods:

- a. Cell growth: Mix 10 mL of culture suspension vigorously with 10 mL ethanol/n-butanol = 1/1 (v/v). After centrifugation at 10,000g (20 min), dry the residual biomass at 105°C for 48 h and estimate the weight.

b. Total nitrogen: After centrifugation of 10 mL culture suspension at 10,000g (20 min), take 0.2 mL supernatant for quantitative measurements according the to operating instructions of Lange's test kit Lato, LCK 338.

- c. Glucose: Buffer 4 mL of above supernatant (b) with 1 mL 1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 7.0) solution, and drop 25 μL on a test strip (see operating instructions of Boehringer-Mannheim; Accutrend alpha).
 - d. Oleic acid: Extract 100 mL culture suspension twice with 100 mL ethyl acetate. Inject 1 μL of the extract (diluted with ethyl acetate to approx 2 g/L oleic acid) onto the GC column. The temperature profile should be as follows: 7 min at 165°C, 1 min 165→170°C, 7 min at 170°C, 5 min 170→220°C. Use oleic acid as standard.
 - e. Sophorose lipids: Use 1–100 μL of above ethyl acetate extract (d) for thin-layer chromatography (silica gel 60 plates). After development, spraying with detecting reagent and heating at 150°C for 1 min (pink/violet spots for glycolipids), take densitometer measurements according to the operational instructions of Desaga. As standard, use isolated sophorose lipid mixture. R_f values: 0.1–0.60.
5. Stop the cultivation after 200 h. After pumping the culture broth into a glass vessel followed by dilution (1/1) with water, the crude product separates at the bottom over night. Take off the aqueous phase (containing the yeast cells), and wash the precipitate with ice-cold water. Remove the aqueous phase again, and dry the drained product under reduced pressure.
 6. To separate the individual sophorose lipids, load the column for medium pressure chromatography with 15 g crude products. Elute the components by 2% steps (900 mL/step) beginning with the highest chloroform/methanol ratio. Evaporate the 300 mL fractions to dryness, and detect with thin-layer chromatography.
 7. Influence of crude glycolipids on the surface tension of water: Dissolve 100 mg in 10 mL dichloromethane. Add 10 vol of this stock solution, from 0.5 to 500 μL , to 20 mL distilled water placed in 10 special vessels. Add in each case pure solvent to reach a final volume of 500 μL throughout. After sonication ($3 \times 1\text{min}$, $T < 40^\circ\text{C}$, rank 4) with, e.g., a sonifier B 30 (Branson, Heusenstamm, GER), measure the surface tension of water at 25°C; use the Autotensiomat (ring method) according to the operational instructions of Lauda/Wobser GmbH & Co. KG.

3.2—

Method 2: Microbial Production of Dodecyl-Sophorosides

1. Sterilize all media at 121°C and 1 bar overpressure for 20 min.
2. For maintaining the yeast strain, see **Subheading 3.1., step 2.**
3. Transfer a loopful of cells into 100 mL seed culture medium placed in a 500-mL Erlenmeyer shake flask (with two inserts). Incubate at 30°C on a rotary shaker (100 rpm) for 48–72 h. Add 10 mL of the seed culture to 500 mL of fermentation medium in a 2-L Erlenmeyer flask (with two inserts). Incubate under above conditions and, after 48, 72, and 96 h, add 8 g/L portions of 2-dodecanol to the microbial culture. Do not adjust the pH.
4. Check the time-course of cultivation by using the following analytical methods:
 - a. Cell growth.
 - b. Glucose: see **Subheading 3.1., steps 4a and 4c.**
 - c. Ammonium ions: After centrifugation of 2 mL culture broth at 10,000g, use the Merckoquant test according to the operational instructions of Merck.

- d. Dodecyl-Sophorosides: Acidify 5 mL whole broth with 1 N HCl to pH 3.0, extract twice with 10 mL ethyl acetate, combine the organic phases, dry over sodium sulfate and subject it to thin-layer chromatography; use 1–100 μ L of solution. After development, spraying with detecting reagent, and subsequent heating at 150°C (1 min), estimate the individual components (R_f values of glycolipids: 0.25–0.63; pink/violet spots with α -naphthol reagent). With vanillin reagent, the residual 2-dodecanol shows blue color.
5. After 210 h, acidify the whole broth with 1 N HCl to pH 3.0, and extract twice with the double volume of ethyl acetate. After drying over sodium sulfate and evaporation of the solvent, remove the residual substrate by washing with cyclohexane. The residue is the final product.
6. Surface tension measurements: see **Subheading 3.1., step 7**, but use an aqueous stock solution of crude glycolipids.

3.3—

Method 3: Microbial Production of Mannosylerythritol Lipids.

1. Sterilize all nutrients—agar, seed, and fermentation media—at 121°C, 1 bar overpressure for 20 min.
2. For maintaining *C. antarctica* CBS 6821 (**Subheading 2.3., item 1**) in your laboratory, incubate the strain on potato-dextrose agar slants at 30°C for 2 d.
3. Inoculate a loopful of cells grown on slants into 300-mL Erlenmeyer flasks containing 30 mL of a seed culture medium. Incubate at 30°C for 2 d on a rotary shaker (220 rpm).
4. Transfer the seed cultures (2 mL) to 20 500-mL Erlenmeyer flasks containing 50 mL of a basal fermentation medium (**Subheading 2.3., item 4**). Incubate at 30°C for 7 d on the rotary shaker.
5. For analytical methods during the cultivation time, do the following each day:
 - a. Measure cell growth in terms of optical density at 580 nm with a Shimadzu-Bausch & Lomb spectronic 20 colorimeter or a similar photometer (water dilution!).
 - b. For determination of glycolipid content, extract 5 mL of emulsified culture broths with 10 mL of ethyl acetate, and subject it either to thin-layer chromatography (method b. i.) or use a test tube assay (method b. ii.).
 - b. i. After development of thin-layer chromatography (**Subheading 2.3., item 5**), spray with detecting reagent, and heat the plate at 110°C for 5–10 min. Estimate the individual components (green/brown) with the densitometer. Standard: pure MELs. R_f values: 0.77 (MEL-A), 0.63 (MEL-B), 0.58 (MEL-C), 0.52 (MEL-D).
 - b. ii. To estimate the concentrations of total MELs, pipet an aliquot (50 μ L) of ethyl acetate extract into a test tube, and evaporate to dryness under reduced pressure. Add 1 mL of distilled water and 5 mL of a 0.2% anthrone solution (**Subheading 2.3., item 6**), and heat the tube for 10 min in a boiling water bath. After cooling, measure the optical density at 620 nm. Standard: a mixture of MELs (weight).

6. At the end of cultivation, combine the culture broths (ca. 1 L), acidify with 6 N HCl to pH 3.0, and then centrifuge at 3000g for 30 min. After precipitation of yeast cells and biosurfactants, wash this precipitate twice with water. After further centrifugation and water separation, the glycolipids (heavy oil) have to be extracted twice with methanol (300 mL). Combine and concentrate the extracts (100 mL), and then wash twice with 2 vol of *n*-hexane to remove any remaining oil and free fatty acids. Now add chloroform (200 mL) and water (100 mL) to the washed concentrate, and shake the mixture. Dehydrate the lower chloroform layer with sodium sulfate, and finally evaporate the solvent to a final crude mixture of mannosylerythritol lipids.

7. For separation of the individual glycolipids, dissolve a quarter of the above syrup in chloroform (5 mL) and place it on a silica gel column (**Subheading 2.3., item 7**). Elute first with 400 mL of chloroform/ethyl acetate (4/1; v/v), then with 800 mL of chloroform/acetone (7/3; v/v), 600 mL of chloroform/acetone (6/4), and 300 mL of chloroform/acetone (5/5). Test the samples for glycolipids.

8. Surface tension measurements: see **Subheading 3.1., step 7**.

3.4—

Method 4: Microbial Production of Trehalose Lipids

1. Sterilize the agar medium at 121°C and 1 bar overpressure for 20 min. Regarding seed/fermentation media, sterilize separately the Fe- (membrane filtration) and *n*-alkane components (121°C, 1 bar overpressure).

2. For maintaining *R. erythropolis* DSM 43215 in your laboratory, incubate the strain on agar slants at 30°C for 48 h.

3. Transfer a loopful of cells into a 500-mL Erlenmeyer shake flask (with two inserts) containing 100 mL of seed culture (do not forget to combine all ingredients sterilized separately). After culturing for 72 h at 30°C and 100 rpm (rotary shaker), inoculate 10 mL of the seed culture to 100 mL of fermentation medium in a 500-mL Erlenmeyer flask. Inoculate in parallel 10 Erlenmeyer flasks with 100 mL fermentation medium. Incubate at the same conditions described before, but after reaching a nitrogen limitation (see ammonium ion determination; ca. 4 d, see **Subheading 2.4., item 6**, and **Subheading 3.4., step 4b**), lower the temperature to 22°C and the pH to 6.0–6.5 (H₃PO₄, 85%). Continue with incubation, and adjust the pH to 6.0–6.5 every day.

4. For analytical methods during the cultivation time, determine daily:

a. Cell growth: Mix 10 mL of culture broth thoroughly with 10 mL ethanol/butan-1-ol/chloroform (10/10/1), and centrifuge at 9600g for 30 min. Wash the pellet with 10 mL water, and dry at 105°C for 48 h. Estimate weight of the pellet.

b. Ammonium ions: After centrifugation of 2 mL culture broth at 10,000g, use the Merckoquant test according the operational instructions of Merck.

c. *n*-Dodecane: Mix 2 mL culture broth with 0.2 mL Ca-acetate solution (20%), 4 mL ethanol, and 2 mL *n*-hexane for 2 min. Centrifuge at 2000 g for 10 min. Isolate the upper layer (*n*-hexane phase), and inject 1 or 2 µL onto the column of a gas chromatograph. Working conditions: nitrogen as carrier gas, injector temperature: 280°C; detector temperature: 280°C; oven temperature/program 50°C→170°C (10°C/min).

d. Trehalose lipids: Acidify 20 mL of culture broth with 10% HCl to pH 3.0, and treat under stirring with 20 mL $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (2/1) for 15 min. After centrifugation for 10 min at 5000 g, separate the organic phase. Repeat the above procedure with the aqueous phase. Combine the organic phases, wash with aqueous 20 mL NaCl solution (120 g/L), and evaporate the solvent under reduced pressure. Dissolve the residue in chloroform, and dry over sodium sulfate. After membrane filtration (0.2 μm) and adjusting the solution volume to 10 mL, subject a certain volume (μL -range) to thin-layer chromatography, spray with detecting reagent, and heat the plate at 150°C for 1 min. Estimate the individual components using the densitometer. R_f values of glycolipids: 0.15–0.60 (pink/violet spots).

5. At the end of cultivation after about 8 d, acidify 500 mL of culture broth with 10% HCl to pH 3.0, and continue with downstream processing as described in the foregoing section, except for the quantitative determinations; use larger volumes corresponding to larger starting volume, and finally evaporate the solvent.

6. Surface tension measurements: see **Subheading 3.1., step 7.**

3.5—

Method 5: Microbial Production of Rhamnose Lipids

1. Sterilize all media at 121°C and 1 bar overpressure for 20 min. Sterilize separately the Fe (membrane filtration), glucose, and *n*-alkane components (121°C, 1 bar overpressure).

2. For maintaining *Pseudomonas* sp. DSM 2874 in your laboratory, incubate the strain on agar slants at 30°C for 30 h and deposit at 4°C.

3. Transfer a loopful of cells into a 500-mL Erlenmeyer shake flask (with two inserts) containing 100 mL of seed culture medium; do not forget to combine all ingredients sterilized separately. After culturing for 72 h at 30°C and 100 rpm (rotary shaker) inoculate 2 mL of the seed culture to 100 mL of fermentation medium in a 500-mL Erlenmeyer flask. Work with five 100-mL cultures in parallel. Incubate at the same conditions described before.

4. Check the time-course of cultivation by using the following analytical methods:

a. Cell growth.

b. Ammonium ions.

c. *n*-Tetradecane: see **Subheading 3.4., steps 4a–4c.**

d. Rhamnose lipids: see **Subheading 3.4., step 4d**, and **Subheading 2.5., item 9**; R_f -values: 0.29, 0.82.

5. After a cultivation time of 7–8 d, acidify 300 mL of culture broth with 10% sulfuric acid to pH 2.0, and extract twice with 300 mL ethyl acetate. Combine the organic phases, and evaporate the solvent under reduced pressure.

6. Surface tension measurements: see **Subheading 3.1., step 7.**

3.6—

Method 6: Microbial Production of Emulsan

1. Sterilize all media at 121°C and 1 bar overpressure for 20 min. Add ethanol (no sterilization) to the 100-mL cultures directly before incubation.
2. For maintaining *A. calcoaceticus* ATCC 31012, incubate the strain on agar slants at 30°C for 24 h, and deposit at 4°C.

3. Transfer a loopful of cells into a 500-mL Erlenmeyer shake flask (with two inserts) containing 100 mL of seed culture (no. 1) medium (do not forget to add ethanol). After 10 h of incubation at 30°C and 100 rpm, inoculate 4 mL to a second 100-mL seed culture (no. 2), which has to be cultivated under the same conditions as mentioned before. Now transfer 10 mL into 250 mL fermentation medium (1-L Erlenmeyer flask with two inserts).

Use five 1-L Erlenmeyer flasks in parallel, and incubate under the conditions described before.

4. Check the time-course of cultivation by using the following analytical methods:

- a. Cell growth: Centrifuge 10 mL of culture broth for 20 min at 9600g, separate the supernatant (deposit for other measurements!), dry the pellet at 105°C for 24 h, and estimate the weight of the pellets.
- b. Ammonium ions: Take above supernatant, and use the Merckoquant test according the operational instructions of Merck.
- c. Ethanol: Take the supernatant of **step 4a** and use the enzymatic test combination according the operational instructions of Boehringer.
- d. Emulsan: Mix 1 mL cell-free supernatant with 5 mL anthrone reagent, and heat for 15 min at 100°C (boiling water bath). Stop the reaction by cooling at 0°C. Measure at 620-nm (blank: 1 mL water and 5 mL anthrone reagent) calibration with isolated Emulsan.

5. After 25 h of incubation (foam formation), separate the cells by centrifugation at 9600 g. Mix 1 L of supernatant with 2 L i-propanol, and stand at 4°C overnight. Separate the supernatant, and redissolve the precipitate (Emulsan) in 150 mL water. Mix once more with i-propanol (300 mL). After precipitation at 4°C overnight, redissolve in 150 mL water, place in a Visking dialysis tube, and dialyze vs 5 L water. After 24 h, freeze-dry the aqueous solution of the tube, and estimate the weight of the residual lipopolysaccharide Emulsan.

6. Surface tension measurements: see **Subheading 3.1., step 7**, but use an aqueous stock solution.

4— Notes

1. Some microorganisms are not currently available from international strain collections (because of patent protection). Instead of, e.g., *R. erythropolis* DSM 43215, purchase *Arthrobacter paraffineus* or *Arthrobacter hydrocarboclastus* strains from culture collections (for example, ATCC, DSM, or CBS). The author is sure you will find trehalose lipids.
2. Equipment of other companies not cited here could be used for measuring, e.g., glucose, lipophilic substrates, and glycolipids.
3. In the extraction of glycolipids and large-scale column chromatography because of hazards, especially for women, chloroform should be replaced by methyl-*t*-butylether or ethyl acetate.
4. Various methods have been described in the literature for detecting and yield determination of glycolipids. All methods may be used for low-mole-wt glycolipids. For total yield, the anthrone method is recommended (because of low solu-

bility, add Brij-56-emulsifier, which is usable also for high-mole-wt Emulsan). For detecting and subsequent estimation of the individual components of low-mole-wt glycolipids, use thin-layer chromatography and subsequently α -naphthol reagent (pink/violet spots) or alternatively anisaldehyde reagent (green/brown spots), before densitometer application. From the author's experience, HPLC determinations are more difficult, but could be developed in the near future.

a. Anthrone reagent: 0.5 g anthrone and 10 g thio-urea in 1 L 72% sulfuric acid. Procedure: Fill 5–20 μ L solution (dependant on the glycolipid content) into a tube and evaporate the solvent. Add 1 mL aqueous 1% Brij-56-solution and 5 mL anthrone reagent, and incubate at 100°C for 15 min. Stop the reaction by cooling at 0°C. After centrifugation at 2000 g for 5 min, measure at 620 nm (blank: 1 mL 1% Brij-56 solution and 5 mL anthrone reagent); calibrate with isolated glycolipids.

b. α -Naphthol reagent: 10.5 mL α -naphthol solution (15% in ethanol), 6.5 mL sulfuric acid (conc.), 40.5 mL ethanol, and 4 mL distilled water.

c. Anisaldehyde reagent: anisaldehyde/sulfuric acid/acetic acid = 0.5/1/50 (v/v/v).

5. Downstream processing is only described for lactonic sophorose lipid and mannosylerythritol lipids, but in principle, these methods are transferable to other microbial glycolipids because of their similar TLC behavior.

6. With shake-flask cultures, often the yields on glycolipids are not as high as reported in the literature. This is owing to insufficient aeration or pH adjustment compared to a bioreactor cultivation.

7. Before measuring surface tension using the tensiomat separate residual oils from lipophilic substrates by chromatography.

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10—

Partial Enzymatic Hydrolysis of Starch to Maltodextrins on the Laboratory Scale

Leon M. Marchal

1—

Introduction

Starch mainly consists of amylopectin (70–80% w/w) and amylose (20–30% w/w). Amylopectin is a branched macromolecule (mol wt 10^7 – $5 \cdot 10^8$) of which on average 1 in 20–25 α -(1,4)-bond glucose units is branched by an α -(1,6)-D-glucosidic bond. Amylose (mol wt 10^5 – 10^7) consists of much longer linear chains of which on average one in several hundred α -(1,4)-bond glucose units is branched by an α -(1,6)-D-glucosidic bond **(1)**. The hydrolysis of starch to low-mol-wt products catalyzed by an α -amylase is one of the most important commercial enzyme processes. The hydrolyzed products are widely used in the food, paper, and textile industry **(2)**. The demand for hydrolyzed starches with specific functional properties has led to the development of a whole range of products. These are commercially available at relatively low prices from major starch producers. The reason to produce hydrolyzed starches on lab scale will therefore mostly be either a kinetic study or the production of a product with a certain defined degree of hydrolysis. The degree of hydrolysis is expressed as dextrose equivalent (DE), which is the percentage of reducing sugar calculated as dextrose on dry-weight basis. The DE of, for example, glucose is 100 (by definition) and starch is virtually zero. Therefore, the dextrose equivalent is a measure of the mean average molecular mass of the starch hydrolysate. Maltodextrins are by definition nonsweet starch hydrolysates with a DE of < 20 **(3)**. Starch hydrolysates with a DE above 20 are classified as glucose syrups. In the industry, maltodextrins are produced at high dry-weight concentrations of 30–40% w/w, with specialized equipment (jetcookers, stirred autoclaves). This equipment will in most cases not be available at the laboratory. For the production of maltodextrins, the suspended starch granules have

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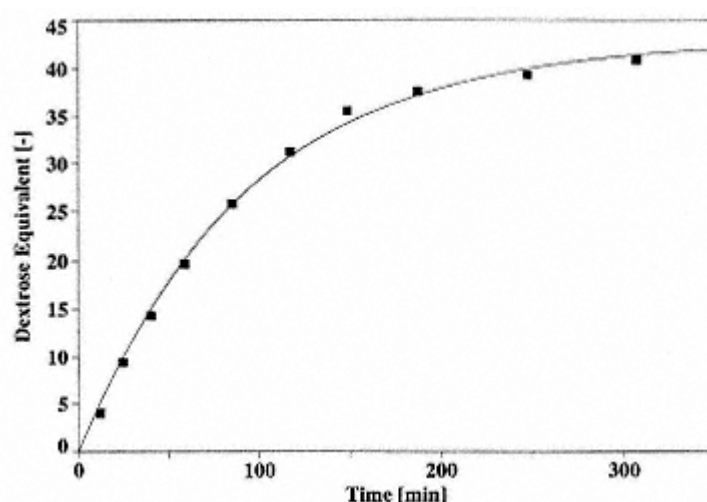


Fig. 1.

Typical DE (as determined by Luff-Schoorl titration) vs time during the hydrolysis of starch with *B. licheniformis* α -amylase.

to be dissolved (gelatinized) by heat treatment, after which α -amylase can be added. α -Amylases can hydrolyze the α -(1,4)-bond glucosidic linkages more or less at random. The average chain length of the starch hydrolysate will decrease as the reaction proceeds. At a DE > 10, the amount of easily hydrolyzable substrate decreases and the reaction rate slows down (see Fig. 1). At a DE of approx 40, the reaction comes virtually to an end. The α -amylase is not able to hydrolyze more α -(1,4)-glycosidic linkages. The mixture then consists predominantly of smaller oligosaccharides (glucose to maltohexaose). Also a considerable fraction (15–25% w/w) of saccharides with a higher degree of polymerization (50–200 glucose units) are present. These are generally described as α -limit dextrins, which originate from the highly branched fractions of the amylopectin (4). To follow the hydrolysis of starch in terms of the dextrose equivalent, several different techniques have been developed (5) (see Note 1). In the industry, two methods are generally used to determine the DE: Lane-Eynon titration (principally used in USA) and Luff-Schoorl titration (principally used in the Western Europe). Here one (Luff-Schoorl titration) is given.

2—

Materials

2.1—

Preparation of Liquefied Starch.

1. Potato or tapioca starch, which can be obtained from one of the many starch producers like Avebe (Veendam, The Netherlands) or Emsland Stärke (Emlichheim,

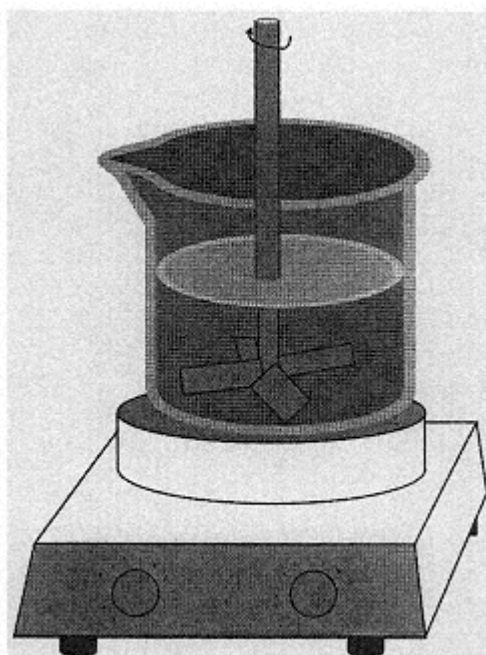


Fig. 2.
Experimental setup for preparing gelatinized starch.

Germany), or from suppliers like Sigma. Starch may be stored at room temperature. There are differences between starches from different botanical sources (see **Note 2**).

2. Heat-stable α -amylase from *Bacillus licheniformis* stored at 4°C (see **Note 3**).

3. pH meter and 0.1 M NaOH and 0.1 M HCl for adjusting the pH.

4. Demineralized water (see **Note 4**).

5. Calcium chloride ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$).

6. Thermometer (range 20–100°C).

7. Beaker with stirrer (see **Note 5** and **Fig. 2**).

8. Aluminum foil.

9. Electric hot plate.

10. Thermostated reaction vessel, with stirrer.

2.2—

Determining the Dextrose Equivalent

1. Luff-Schoorl reagent: Mix the following salts well in a dry glass beaker: 86.2 g anhydrous sodium carbonate (Na_2CO_3), 31.3 g sodium hydrogen carbonate (NaHCO_3), 70.0 g citric acid, trisodium salt dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$), 25.0 g copper(II)sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). Dissolve (in small portions) under continuous stirring in 800 mL demineralized water. The solution is dark blue. Transfer to a measuring glass, and fill to 1 L with water. To check the reagent, dilute 20 mL

to 1 L with water. The pH should be between 9.8 and 10.2. Luff-Schoorl reagent can be stored (at room temperature) for at least a year, but check before use (see **Note 6**).

2. 1 M potassium iodide (store in a dark bottle).

3. 2.5 M sulfuric acid. Add slowly 140 mL of concentrated sulfuric acid (96%) to 700 mL water. Let it cool and make up to 1 L.

4. Sodium thiosulfate volumetric standard, 0.1 N solution in water. Store in a brown bottle.

5. Starch indicator, 1% solution. Add 10 g of light oxidized starch (for example, Perfectamyl A-5760, Avebe, Veendam, The Netherlands) to 1 L boiling water. Cool and add three drops of chloroform (to prevent microbial growth).

6. 1 M potassium thiocyanate.

7. Antifoaming agent (for example, *n*-Octanol).

8. Boiling aid (for example, glass beads).

9. Heating device.

10. Boiling flask (Erlenmeyer) with a reflux condenser at least 25 cm in height.

11. Heat-insulated gloves.

12. Stopwatch.

13. Burette, with a capacity of at least 25 mL, graduated in subdivisions of 0.05 mL.

14. Balance.

3—

Methods

3.1—

Preparation of Liquefied Starch

1. Suspend the starch well in demineralized water. Maximum starch concentration recommended for this procedure is 2–5% by dry weight (see **Notes 7** and **8**).

2. Add approx 100 ppm of calcium (see **Note 9**).

3. Adjust the pH to 6.6. Take care to keep the starch granules in suspension, and allow up to 15 s in a well-mixed system for the pH to come into equilibrium (see **Note 10**).

4. Heat the starch suspension from room temperature to 100°C in about 0.5 h under continuous stirring. Mix especially well when the starch starts to gelatinize (above 60°C). Enhance the stirring speed if the boiling bubbles seem to be stuck at the bottom of the beaker. Take care not to lose too much water through evaporation (cover the beaker with aluminum foil).

5. Boil for another 5–10 min.

6. Transfer the now viscous solution into a reaction vessel, and allow the starch solution to cool to the desired reaction temperature (85°C). Slow stirring will prevent the formation of skins on the starch solution. Gelatinized starch should be used as soon as possible, since irreversible retrogradation will occur when stored (see **Note 11**).
7. Adding 0.05–0.1% of enzyme preparation on starch dry weight basis will start the hydrolysis. The viscosity will decrease rapidly as the reaction proceeds.
8. After 5–10 h the hydrolysis reaction will virtually come to an end, since the enzyme cannot hydrolyze more α -1,4 bond glucose units. The corresponding DE is approx 40. See **Subheading 3.2.** when you want to monitor the reaction (to stop at a desired lower DE).

9. To inactivate the enzyme, adjust the pH to 3.5–4.0 and heat for 10 min at 95°C (6). Adding a calcium-complexing agent will facilitate the inactivation of the enzyme (see **Note 12**).
10. Coagulated protein can be removed by filtration through a normal paper filter (see **Note 13**).
11. Hydrolysates with a DE between 15 and 40 can be stored at 4°C or frozen. Lower maltodextrins (<DE 15) are unstable when stored for a longer time in a liquid form (see **Note 14**).

3.2—

Determining the DE

1. To determine the amount of sodium thiosulfate used in a blank, add 25 mL of Luff-Schoorl reagent to 75 mL demineralized water in a 300-mL Erlenmeyer. Do not boil this solution, and perform **steps 10–14** as below. A blank usually uses around 25 mL of sodium thiosulfate (0.1 N).
2. Put in a 300-mL Erlenmeyer approximately the following amount of starch solution (weigh on a balance) (see **Note 15**):

$$35 \times (100/\text{dry-wt percentage}) \times (100/\text{DE}[\text{estimated}]) \times \text{mg} \quad (1)$$

Record the exact amount of (mg) of starch solution used.

3. Add 25 mL of demineralized water.
4. If the sample is not in the neutral range (pH 6.0–8.0) neutralize.
5. Add 25 mL of Luff-Schoorl reagent and some boiling aid (for example, glass beads).
6. Put the Erlenmeyer, fastened to a Re/Lux condensator, on an electric hotplate.
7. The solution has to cook within 2 min. Then cook for exactly 10 min (± 10 s). If excessive foaming occurs, add a few droplets of the antifoaming agent.
8. Flush 50 mL of demineralized water through the cooler. Take the Erlenmeyer off the electric hotplate (use gloves; the Erlenmeyer will be hot).
9. Cool the solution fast to room temperature in a cold water bath or under a running tap.
10. Add subsequently: 20 mL of potassium iodide (1 M) and 25 mL of sulfuric acid (2.5 M). (CO₂ emission) **Add the sulfuric acid slowly!**
11. Titrate this solution under continuous stirring with sodium thiosulfate (volumetric standard, 0.1 N) from dark brown to light brown.
12. Add 5 mL of starch indicator. The solution will become dark blue to purple.
13. Addition of 5 mL potassium thiocyanate gives a sharper end point of the titration. Titrate the solution until creme white.
15. Subtract the amount of used sodium thiosulfate from a blank (no starch present). Look up this value in **Table 1**. If the titration difference (blank—sample) is < 1 mL, do the above procedure over again with more sample (starting at **step 2**).
16. The DE can be calculated from:

$$\text{DE} = [\text{Value Table 1}/(\text{Dry wt percentage} \times \text{amount of sample (mg)})] \times 100 \quad (2)$$

4—

Notes

1. A disadvantage of these titration methods (Lane and Eynon or Luff-Schoorl) is their overestimation of the exact DE. This overestimation varies for different

Table 1
Values for Determining the Luff-Schoorl DE^a

	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	0.01	0.02	0.03	0.04	0.05	0.06	0.07
1	240	264	288	312	336	360	384	408	432	456	1	5	7	10	12	14	17
2	480	504	528	552	576	600	624	648	672	696	2	5	7	10	12	14	17
3	720	745	770	795	820	845	870	895	920	945	2	5	8	10	13	15	18
4	970	995	1020	1045	1070	1095	1120	1145	1170	1195	3	5	8	10	13	15	18
5	1220	1245	1270	1295	1320	1345	1370	1395	1420	1445	3	5	8	10	13	15	18
6	1470	1495	1520	1545	1570	1595	1620	1645	1670	1695	3	5	8	10	13	15	18
7	1720	1746	1772	1798	1824	1850	1876	1902	1928	1954	3	5	8	10	13	16	18
8	1980	2006	2032	2058	2084	2110	2136	2162	2188	2214	3	5	8	10	13	16	18
9	2240	2266	2292	2318	2344	2370	2396	2422	2448	2474	3	5	8	10	13	16	18
10	2500	2526	2552	2578	2604	2630	2656	2682	2708	2734	3	5	8	10	13	16	18
11	2760	2787	2814	2841	2868	2895	2922	2949	2970	3003	3	5	8	11	14	16	19
12	3030	3057	3084	3111	3138	3165	3192	3219	3246	3273	3	5	8	11	14	16	19
13	3300	3327	3354	3381	3408	3435	3462	3489	3516	3543	3	5	8	11	14	16	19
14	3570	3598	3626	3654	3682	3710	3738	3766	3794	3822	3	6	8	11	14	17	20
15	3850	3878	3906	3934	3962	3990	4018	4046	4074	4102	3	6	8	11	14	17	20
16	4130	4159	4188	4217	4246	4275	4304	4333	4362	4391	3	6	9	12	15	17	20
17	4420	4449	4478	4507	4536	4565	4594	4623	4652	4681	3	6	9	12	15	17	20
18	4710	4739	4768	4797	4826	4855	4884	4913	4942	4971	3	6	9	12	15	17	20
19	5000	5030	5060	5090	5120	5150	5180	5210	5240	5270	3	6	9	12	15	18	21
20	5300	5330	5360	5390	5420	5450	5480	5510	5540	5570	3	6	9	12	15	18	21
21	5600	5631	5662	5693	5724	5755	5786	5817	5848	5879	3	6	9	12	16	19	22
22	5910	5941	5972	6003	6034	6065	6096	6127	6158	6189	3	6	9	12	16	19	22
23	6220	6251	6282	6313	6344	6375	6406	6437	6468	6499	3	6	9	12	16	19	22

^aLook up in the vertical column the amount of sodium thiosulfate used in the blank—sample in mL. Then look up in the first horizontal column the tenths of mL. Add to this number the value given for the use of hundreds of mL sodium thiosulfate in the right part of the table. For example, 25 mL (blank)—14.74 mL (sample) = 10.26 mL, the corresponding value is 2552 + 16 = 2568.

saccharides, which makes it hard to correct for this (7). A fast (2 min) and accurate alternative to titration methods is measurement the osmolality of the solution with freezing-point depression. The mean average molecular weight of a starch solution can be determined by dividing the amount of solute present (per kg of water) by the osmolality. From this mean average molecular weight, a theoretical DE can be calculated:

$$\text{DE} = (180/\text{Mean mole wt starch hydrolysate}) \times 100$$

Since salts present at the start of the liquefaction also contribute to the osmolality, the measured osmolality has to be corrected for this. Measuring the osmolality of the gelatinized starch solution before liquefaction is difficult owing to the high viscosity. The initial reaction of α -amylase is zero-order. The contribution of salts to the osmolality can therefore be determined by extrapolating the curve to $t = 0$. More information is given in ref. (8).

2. Potato or tapioca starch is preferred above other starches (corn, wheat) because of the lower levels of impurities (lipids, protein). Potato and tapioca starch have a lipid and protein contents of 0.1% on dry-wt basis. Corn and wheat starches have lipid contents of 0.7 and 0.8 and protein contents of 0.35 and 0.4%, respectively. These high levels lead to the undesired formation of amylose-lipid complexes and color formation during liquefaction. Wheat, corn, and tapioca starch have a lower peak viscosity than potato starch (9). This can be an advantage to achieve higher dry-wt concentrations at laboratory scale (see Note 8).

3. Liquid enzyme extracts from the major enzyme producers Novo (Bagsvaerd, Denmark) or Genencor (Rochester, NY) are sufficiently pure. *B. licheniformis* α -amylase (optimum temperature 85°C) is produced by Novo (Termamyl®) and Genencor (Spezyme AA®). α -Amylase from *Bacillus amyloliquefaciens* is somewhat less heat-stable (optimum temperature 70°C) and can be obtained from Novo (BAN®). In addition to differences in temperature stability, these two enzymes also differ slightly with respect to the composition of major end products. The major end products (on weight basis) of *B. licheniformis* α -amylase are maltopentaose (G5), maltose (G2), and maltotriose (G3). The major end products of *B. amyloliquefaciens* α -amylase are G6 (maltohexaose) and G2, G3, and G5.

4. α -Amylases are very sensitive to certain heavy metals. Using tap water can cause inhibition even when small amounts of copper are present.

5. A stirrer that covers a relative large portion of the beaker should be used. A magnetic stirrer is not sufficient. The peak viscosity of a 5% (w/w) potato starch solution is about 3000 Brabender units (≈ 6000 mPa.s) (9). The convection in the solution is very low owing to this high viscosity, and a relative small stirrer will only mix part of the beaker well. An axial-flow impeller (for example, an axial flow four-blades or a marine propeller) will enhance the exchange between the heated bottom and the rest of the beaker, thus minimizing large temperature differences. Use a wide beaker (large heating surface), and do not fill the beaker with too much starch suspension. A large distance between the bottom of the beaker and the liquid surface will enhance the chances of burned starch.

6. To check the Luff-Schoorl reagent, the DE of dextrose (D-glucose) can be measured. Follow the procedure as described in **Subheading 3.2**. If the DE is outside the region 99–101, new Luff-Schoorl reagent should be made. This standardization (in duplicate) should be done for each new batch of Luff-Schoorl reagent.

7. Commercial potato starch usually has a moisture content of $\approx 18\%$. Corn, wheat, and tapioca starches have a moisture content of $\approx 13\%$. These contents may vary owing to differences in relative humidity. The water content may be determined from the loss on drying (90 min at 130°C). This should not be done with starch to be used for experiments, since heat treatment will irreversibly change the structure of the starch (heat-moisture treatment).

8. A higher starch concentration will lead to a higher viscosity of the gelatinized starch solution. At a certain concentration $\pm 5\%$ (w/w), potato starch will not gelatinize completely. Moreover, the viscosity becomes so high that boiling bubbles cannot leave the bottom of the beaker. This leads to increased heat and burning of the starch. The problems at high starch concentration may be overcome by adding the α -amylase before heating. Owing to the α -amylase activity, the viscosity will be reduced. Dry-weight concentrations of 20% (w/w) are quite possible this way. A disadvantage is that this procedure cannot be used for kinetic studies. In addition, some enzyme inactivation may occur when boiling.

9. Addition of calcium will increase α -amylase stability. For most bacterial α -amylases, 100 ppm ($\mu\text{g/g}$ suspension) calcium are sufficient for maximum stabilization.

10. In this procedure, the pH is adjusted before gelatinization. Adjusting the pH after gelatinization gives practical problems owing to the high viscosity of gelatinized starch. Adjusting the pH should not be done with a concentrated solution of base, since this causes unwanted chemical gelatinization. A buffer can be used. However, there are a lot of indications in the literature that buffers influence the α -amylolytic hydrolysis of starch (**10–12**). When the objective is to prepare a defined maltodextrin, this is no problem. For kinetic studies, it may affect results. During the gelatinization, the pH will drop somewhat mainly because of dissolving of CO_2 . Especially potato starch (owing to starch phosphate mono-esters) has some buffering capacity, and the pH will be around pH 6.0 after gelatinization.

11. Gelatinized starch will have a tendency to retrograde. This time-dependent physical phenomenon is, among other factors, enhanced by low temperature (below 70°C). Therefore gelatinized starch should be used immediately for liquefaction.

12. Adding strong calcium-complexing agents, such as EDTA, will strongly increase the inactivation of the enzyme (**13**). Adding 1 mM of ZnSO_4 strongly inhibits the α -amylase activity (**14**). Adjusting the pH to ≈ 3 will also stop the enzyme activity, but will not irreversibly inactivate the enzyme.

13. At higher temperatures (at neutral pH usually above 70°C), the proteins in the present starch will coagulate and form small white precipitates during liquefaction. This coagulation is stronger at higher DE (lower viscosity). The thermal enzyme inactivation step (**9**) increases the protein coagulation. When the enzyme is not thermally inactivated, protein coagulation can be enhanced by adjusting the pH to 4.8 and heating for 20 min at 80°C .

14. The best way to store low-DE maltodextrins for prolonged times is to dry them. On an industrial scale, this is done by spray-drying.

15. During a hydrolysis, each time a glycosidic linkage is hydrolyzed, water is added to the hydrolysate and the mass of the solute increases. When starch, for example, is hydrolyzed to a Luff-Schoorl DE of 40, the mass increases with $\approx 3\%$ **(8)**. A convenient and fast way to check the dry-weight content of a starch hydrolysate during liquefaction is by measuring the refractive index. The refractive index is based on the optical rotation of the individual sugars. Since the optical rotation is dependent on the type of sugar, dry matter content thus determined deviates somewhat from the actual one. The exact dry-weight content is determined by drying in an oven. To suppress Maillard reactions, drying at 100°C for 4 h at reduced pressure is recommended.

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11—**The Production of $\alpha(1\rightarrow2)$ -Terminated Glucooligosaccharides**

Marguerite Dols, Vincent Monchois, Magali Remaud-Siméon, René-Marc Willemot, and Pierre F. Monsan

1—**Introduction**

Oligosaccharides are molecules of biological interest because of their specific role in recognition mechanisms **(1)**, a property directly linked to the complexity of the structure of their carbohydrate units. These molecules are difficult to produce by chemical synthesis because this requires complex successive protection and deprotection steps **(2)**. At present, three routes are commonly recognized for oligosaccharide production **(3–5)**:

1. Extraction from plant source.
2. Controlled enzymatic hydrolysis of polysaccharides.
3. Enzymatic synthesis.

Glucooligosaccharides (GOS) belong to a variety of oligosaccharides that are enzymatically synthesized at high yields by glucosyltransferases from *Leuconostoc mesenteroides* (EC: 2.4.1.5). These enzymes synthesize from sucrose substrate **(6)** a high-mol-wt glucopolysaccharide named dextran. In the presence of an acceptor, such as maltose, they produce a series of oligosaccharides of increasing molecular weight, without the need for activated sugars or cofactors **(7–9)**.

Dextran is formed by a main chain of α -D-glucopyranosyl units linked through $\alpha(1\rightarrow6)$ linkages with $\alpha(1\rightarrow2)$, $\alpha(1\rightarrow3)$, or $\alpha(1\rightarrow4)$ branch linkages **(10)**. The structure of the polymer is highly dependent on the enzyme-producing strain **(11,12)**. Because of its various applications (as chromatographic support, plasma substitute, and so forth), the dextran produced by dextranase from *L. mesenteroides* NRRL B-512F is the most studied. Recently, *L. mesenteroides*

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NRRL B-1299 dextranucrase, the only glucosyltransferase known to produce a dextran with a high proportion of the rare $\alpha(1\rightarrow2)$ linkage (**11,13**), was used industrially to synthesize $\alpha(1\rightarrow2)$ terminated GOS from sucrose and maltose, low-cost and renewable substrates (**14**). These molecules withstand the attack by digestive enzymes of humans and animals, and are selectively consumed by bacteria of the intestinal microflora, such as *Bifidobacterium* sp., *Lactobacillus* sp., or *Bacteroides* sp. This prebiotic effect is connected with the induction of a large variety of glycosidase and may prevent the development of pathogenic flora (**15**). Because of this prebiotic property, GOS are already marketed for dermo-cosmetical applications, and may be used in human or animal feed (**5**).

This chapter is dedicated to the operational procedure used to obtain $\alpha(1\rightarrow2)$ -terminated oligosaccharides. First, it provides the protocol for the production and purification of the specific dextranucrase that catalyzes the synthesis of $\alpha(1\rightarrow2)$ linkage. Next, it focuses on the protocol for the in vitro GOS synthesis, and gives the analytical method used to characterize the various products and evaluate the reaction yields. Finally, the last section is devoted to the purification of GOS on a preparative scale.

2—

Materials.

1. *L. mesenteroides* NRRL B-1299 cells (supplied by the N.R.R.L., Peoria, IL) are stored frozen in standard medium (see **step 4**) with 18% (w/v) glycerol at -18°C .

2. Stock solutions for culture medium:

- a. Phosphate buffer: K_2HPO_4 , 200 g/L, pH 6.9, adjusted with orthophosphoric acid.
- b. 200 g/L Yeast extract (bioMérieux).
- c. 200 g/L Sucrose.

These solutions (prepared in distilled water) and 1 L of distilled water are sterilized separately by autoclaving for 20 min at 120°C (see **Note 1**), and can be stored for 1 mo at room temperature.

3. Micro elements (20 g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$; 1 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; 1 g/L NaCl, 2 g/L CaCl_2 and 2 g/L $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$) must be dissolved separately in distilled water and then mixed together (see **Note 2**). The final preparation is sterilized by filtration (0.2- μm cutoff). A red coloration sometimes appears during storage (1 mo at room temperature) because of Fe oxidation.

4. The standard culture medium (500 mL) is obtained by mixing 100 mL sucrose solution, 50 mL of yeast extract, 50 mL of K_2HPO_4 and 10 mL of micro-element solution and 290 mL of distilled water (see **Note 3**). This complete medium can be stored at $+4^{\circ}\text{C}$ for up to 3 d before inoculation.

5. For enzyme fractionation, prepare 300 mL of polyethyleneglycol 1500 (50%, w/v in ultrapure water) (see **Note 4**). This preparation can be stored for 2 wk at $+4^{\circ}\text{C}$.

6. Enzyme buffer: prepare 1 L of sodium acetate buffer (200 mM, pH 5.4) and 1 L of salts (CaCl_2 0.5 g/L + NaN_3 10 g/L) in ultrapure water. These concentrated ($\times 10$) solutions can be stored for 1 mo at $+4^{\circ}\text{C}$.

7. Resuspension buffer (400 mL) is obtained by mixing 40 mL of enzyme buffer and 40 mL of salts ($\text{CaCl}_2 + \text{NaN}_3$) with 320 mL ultrapure water.
8. For standard activity measurements and GOS synthesis, prepare 200 mL of sucrose (400 g/L) and 100 mL of maltose (400 g/L) in ultrapure water. These concentrated solutions can be stored for up to 1 wk at 4°C, and up to 6 mo at -18°C.
9. DNS reagent: mix completely 5 g 3,5-dinitro-salicylic acid with 8 g NaOH and 300 mL ultrapure water. Add 150 g of Na, K tartrate, complete volume to 500 mL with ultrapure water and mix until complete dissolution of the salt. The reagent is light-sensitive, so keep the bottle covered with aluminum paper. DNS can be stored for several months.
10. GOS enrichment: the enzymes used are *A. niger* glucoamylase (NOVO NORDISK) and *Penicillium* sp. endodextranase (AMANO) (see **Note 5**). They must be diluted just before reaction.
11. HPLC analysis: reverse-phase chromatography (C18 column, Ultrasep 100, 6 μm , 5 $\times$ 300 mm, Bischoff chromatography) is carried out using a Hewlett Packard 1050 series system consisting of a pump, an injector, and an HP 1047A refractometer. The recommended injection volume is 20 μL and the total sugar concentration in the sample should not exceed 5 g/L.

3—

Methods

Four points are developed in this section. First, the methods to produce, fractionate, and concentrate the specific enzyme involved in the reaction are presented. Then, we focus on the enzymatic reaction procedures, and we finish with the analytical aspects.

3.1—

Dextransucrase Production

1. Inoculation: Erlenmeyer flasks containing 500 mL of the standard medium (see **Note 6**) are inoculated at 2% (v/v) with a tube containing frozen cells.
2. Incubation: the Erlenmeyer flasks are incubated on a rotary shaker at 200 rpm and 25–27°C (**Note 7**), for 7–8 h. Cell growth can be followed as described in **Note 8**.

3.2—

Enzyme Fractionation

The global procedure for enzyme fractionation (see **Note 9**) and concentration is summarized in **Fig. 1**.

1. Harvesting: when the optical density stabilizes around 18–20, after 7 h of culture, the culture medium is cooled to 4°C and the pH adjusted to 5.6 to favor enzyme stability.
2. Centrifugation: the two forms of *L. mesenteroides* NRRL B-1299 dextransucrase are separated by centrifugation of 500 mL of culture broth at 7000g +4°C, for 30 min.
3. Dextransucrase insoluble preparation (IGT): the pellet, containing the insoluble dextransucrase (IGT), is washed with 500 mL of acetate buffer (20 mM, pH 5.4): it is re-suspended by vigorous agitation and centrifuged at 7000g, +4°C, for 30 min; then the pellet is diluted in 200 mL of resuspension buffer (see **Note 10**).

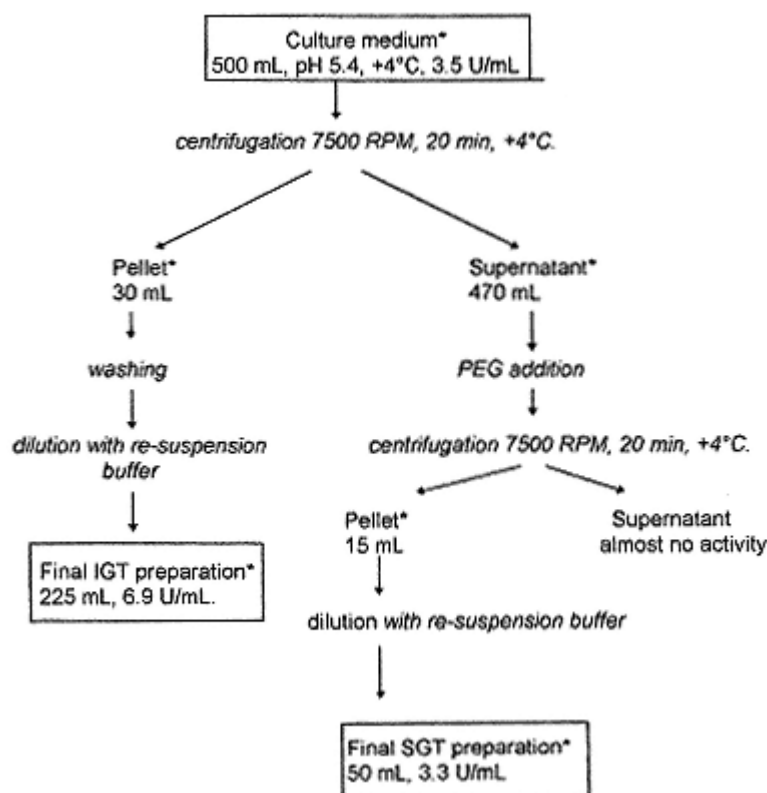


Fig. 1.

Schematic representation of the dextransucrase fractionation procedure.

*Preparations that contain dextransucrase and that can be used for measuring standard enzyme activity.

4. The soluble form (SGT) is concentrated from the supernatant by means of aqueous two-phase partition between dextran and polyethylene glycol (PEG) 1500 (16). PEG is added dropwise until a white coloration appears owing to dextran and dextransucrase precipitation. The dextran phase, harvested in the pellet by centrifugation at 7000g, +4°C, for 30 min, is diluted in 35 mL of re-suspension buffer.

5. Conservation: SGT and IGT concentrated preparations can be stored for up to 1 wk at 4°C and more than 5 mo at -18°C, without significant loss of activity. Moreover, the presence of NaN_3 avoids any possible contamination.

3.3—

Dextransucrase Standard Activity Measurements (see Note 11)

1. Dextransucrase preparation is diluted in the following medium (total reaction volume 5 mL), previously incubated at 30°C: sodium acetate buffer 20 mM,

pH 5.4, 100 g/L sucrose, 0.05 g/L CaCl_2 and 1 g/L NaN_3 . This corresponds to time 0 of the kinetic measurement.

2. The reaction medium is incubated at 30°C for 30 min under magnetic stirring. 0.5 mL samples are taken from the reaction medium at regular time intervals (1, 5, 10, 15, 20, and 30 min), and immediately mixed with 0.5 mL DNS reagent, which stops the reaction.

3. 0.5 mL of blank (ultrapure water) and various references (containing from 0.2–2 g/L fructose) are also mixed with 0.5 mL DNS reagent.

4. All the samples and references are heated at 90°C for 5 min and cooled to room temperature before ultra-pure water addition (5 mL). The optical density is read at 540 nm. One unit of dextranucrase activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of fructose/min under the conditions for standard assay. The method sensitivity is about 0.05 U/mL for dextranucrase activity in the assay tube.

3.4—

GOS Synthesis

GOS synthesis catalyzed by *L. mesenteroides* NRRL B-1299 dextranucrase is described precisely in Note 12. This section first indicates the reaction conditions necessary for GOS synthesis. Then it concentrates on various optional purification (**steps 4 and 5**) and enrichment (**step 6**). These optional procedures must be done in the same order as they appear here (**steps 1–7**).

1. Reaction: the reaction medium (10 mL) is composed of sucrose 100 g/L, maltose 33 g/L, acetate buffer (20 mM, pH 5.4), CaCl_2 0.05 g/L, and NaN_3 1 g/L. The enzyme concentration required for complete reaction within 10 h is 1 U/mL IGT or 0.3 U/mL SGT (see **Note 13**).

2. Reaction is carried out at 25°C under magnetic stirring.

3. End of the reaction: after 8–10 h, the reaction is stopped by heating the tubes at 90°C for 10 min (see **Note 14**).

4. Elimination of dextran (see **Note 15**): the GOS mixture is centrifuged for 5 min at 14 000g, room temperature, and then ultrafiltered using an Amicon hollow fiber system (membrane cutoff = 100,000).

5. Elimination of fructose: the GOS preparation obtained in 4 contains about 50 g/L fructose and 70 g/L of a mixture of maltose, $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow6)$ GOS. Fructose can be removed by chromatography on a strongly acidic column (Duolite C-204F) loaded with calcium.

6. Enrichment in $\alpha(1\rightarrow2)$ GOS: the mixture can be enriched in $\alpha(1\rightarrow2)$ GOS by incubation with endodextranase (20 U/mL) or glucoamylase (3 AGU/mL) (see **Note 6**).

- Reaction is carried out at 37°C, pH 5.4 (acetate buffer, 20 mM), with the GOS solution obtained in **step 5** diluted by half.

- Reaction is nearly complete within 6 h, but it is allowed to continue for 15 h and stopped by heating the tubes at 90°C for 10 min.

7. All the GOS preparations (obtained in **steps 3, 4, 5 and 6**) can be stored a week at 4°C and several months at -18°C.

3.5—

HPLC Analysis

1. GOS are separated according to their size and structure by HPLC on a C18 column and eluted with 0.5 mL/min ultrapure water at room temperature (20–25°C). Products are detected by means of their refractive index with a refractometer. Typical chromatograms of the various preparations obtained are described in **Note 16**.
2. The relative concentration of each molecule can be evaluated by calibration of the refractometer with maltose, sucrose, and fructose (0.5, 1, 2.5, and 5 g/L each) as references.
3. The apparent yield in oligosaccharides can be calculated by the following equation (see **Notes 17** and **18**):

$$\text{yield} = \frac{100 \times \text{Sum (final oligosaccharide concentrations)}}{(\text{maltose} + 0.474 \text{ sucrose}) \text{ initial concentrations}}$$

4—

Notes

1. The various components are sterilized separately to avoid Maillard reactions.
2. The salt powders should not be mixed together before adding water. Otherwise, precipitation of most of the components will occur. In addition, care must be taken to mix the various products in the order indicated here.
3. A recent study of the medium composition was made to find the minimal concentration necessary for each component (**17**). Except for certain salts, all the elements in the medium were found to be essential for dextransucrase production. During the culture, sucrose is used by the bacteria as a carbon source for growth, but also as the dextransucrase inducer (**18**). Yeast extract provides the nitrogen components and vitamins necessary for growth. The phosphate buffer is used to slow down the pH fall owing to lactic acid and acetic acid production from sucrose metabolism by *L. mesenteroides* (**19,20**). A pH range between 6.5 to 6.9 is advised as the best compromise between enzyme stability and a cell physiology compatible with dextransucrase production (**19,21**).
4. After storage at low temperature (+4°C), standard medium must be heated to at least room temperature and at most 27°C before inoculation.
5. Do not use a higher polymerization degree than 1500, to avoid the precipitation of products other than dextran and dextransucrase.
6. Both enzymes convert the α(1→6) GOS into glucose residues. One unit of glucoamylase corresponds to the amount of enzyme that hydrolyzes 1 μmol of maltose/min at 25°C, pH 5.4 (acetate buffer 20 mM) in the presence of 100 g/L maltose. One unit of endodextranase corresponds to the amount of enzyme that produces 1 μmol of isomaltose/min from linear T70 dextran (Sigma, St. Louis, MO, 50 g/L) at 37°C and pH 6.0 (20 mM K₂HPO₄ buffer).
7. 27°C is the recommended temperature for the best compromise between rapid growth and enzyme stability; enzyme production is also possible at lower temperatures (20–25°C), but it is inadvisable to use a culture temperature higher than 27°C (**21,22**).

8. Cell growth can be followed by measuring the absorbance at 650 nm. The optimum dilution range (distilled water) is between 5 and 20. After about 6 h, it reaches quite high values: about 20 absorbance units. These high values result from cell multiplication but also from dextran formation from sucrose owing to the extracellular dextranase activity. For additional details on enzyme production, see Dols et al. (17,20).

9. *L. mesenteroides* NRRL B-1299 dextranase is mainly insoluble: 90% of the enzyme is recovered in the pellet after centrifugation (23). Many unsuccessful attempts have been made to increase the enzyme solubility (9,24). Moreover, during batch or fed-batch cultures in stirred reactors, the proportion of the soluble form of dextranase remains unchanged even when it is possible to increase the level of activity produced significantly (17). Nevertheless, both soluble and insoluble dextranase produce $\alpha(1\rightarrow6)$ GOS (9), but the respective protocols necessary to concentrate each form of dextranase are different and thus they are generally used separately. The protocol described in **Subheading 3.2.** enables a standard activity recovery of 98% from the culture medium. The final SGT preparation contains soluble dextran associated with dextranase, but the insoluble preparation contains cells and insoluble dextran. Consequently, NaN_3 is used to avoid cell metabolism in the presence of substrates, but it does not affect dextranase activity. No significant interfering activity (levanase, invertase, or sucrose-phosphorylase) can be detected in the two dextranase fractions produced by *L. mesenteroides* NRRL B-1299 (20).

10. Resuspension can be made easier by means of an Ultra Turrax (IKA ULTRA TURRAX ®T25, Janke & Kunkel, Labortechnik Staufen) mixer.

11. Standard activity measurements are carried out in order to measure the active enzyme content of either the culture medium or the final SGT and IGT preparations. They consist of measuring the velocity of fructose formation from sucrose during dextran synthesis by dextranase (6). Fructose is assayed as a reducing sugar by the method of Sumner and Howell (25). If, for dextranase production, the advised pH value is around 6.7 (see **Note 3**), that for both enzyme purification and activity measurements is 5.4 (23).

12. In the presence of sucrose and maltose (acceptor molecule), dextranase catalyzes the transfer of glucosyl units to the nonreducing end of the acceptor (**Fig. 2**). The fructose produced accumulates in the medium or can act as an acceptor. The first product of the reaction on maltose is panose (6-O- α -D-glucosyl maltose). Panose is also an acceptor for dextranase resulting in an increase in molecule length and a higher mol-wt GOS (8). Beyond the degree of polymerization 4, both $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow2)$ bonds are formed. All the GOS bearing only $\alpha(1\rightarrow6)$ linkages (except the $\alpha[1\rightarrow4]$ bond coming from maltose) are easily digestible by glucoamylase; they belong to the OD (oligodextran) family. On the other hand, the $\alpha(1\rightarrow2)$ GOS withstand hydrolysis by such enzymes; they were called R, for resistant to hydrolysis (9). As shown in **Fig. 2**, dextran and leucrose are side-products of the reaction. Dextran polymer is directly synthesized from sucrose, whereas leucrose (5-O- α -D-glucosyl D-fructopyranose) is the result of an acceptor reaction on fructose (26).

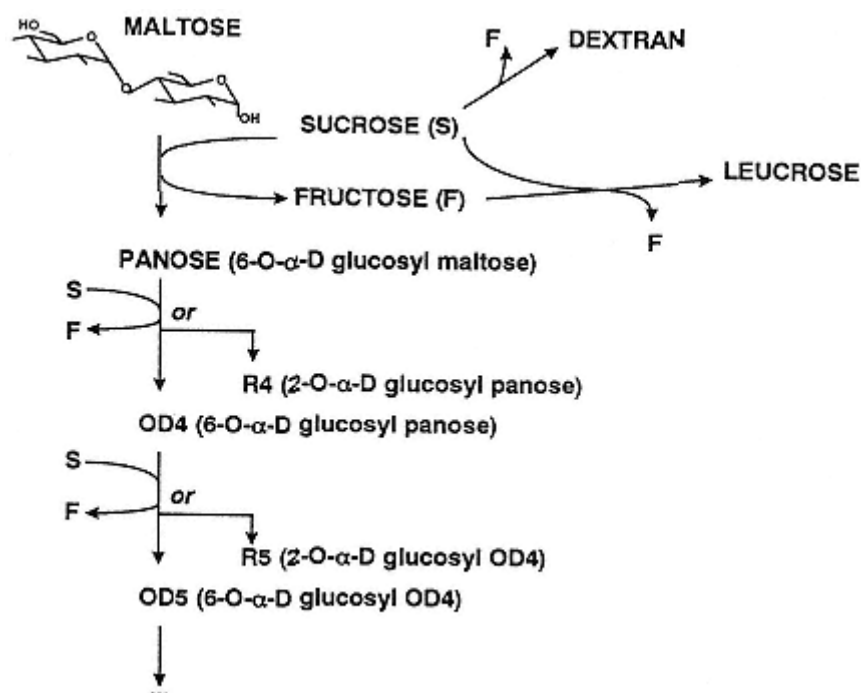


Fig. 2.

Schematic representation of the various reactions catalyzed by *L. mesenteroides* NRRL B-1299 dextranase in the presence of maltose and sucrose.

13. Owing to its specific dextran content, SGT is three times more active than IGT in the presence of maltose, and it can be introduced in lower quantities into the reaction mixture (9,23).

14. If the reaction lasts more than 20 h, the composition of the oligosaccharide preparation obtained can be modified because of disproportionation reactions (27).

15. This protocol enables the elimination of the catalyst and both the insoluble and the soluble endogenous dextran, which would damage the HPLC columns.

16. The chromatogram of the oligosaccharide preparation (obtained after fructose elimination) is presented in Fig. 3A. The first peak eluted corresponds to fructose. The second one corresponds to maltose, which cannot be separated from leucrose, unless leucrose and maltose are present in equimolar quantities in the mixture. Just after this second peak, a third one could indicate the presence of remaining sucrose. Next, comes the peak of panose, followed by a series of GOS of degree of polymerization higher than 4.

Among these molecules, the $\alpha(1\rightarrow2)$ GOS can be identified by comparison with the chromatogram of the glucoamylase treated GOS (Fig. 3B). These GOS are named Ri in Fig. 3, i being the degree of polymerization of the GOS, and the $\alpha(1\rightarrow6)$ GOS are named ODi.

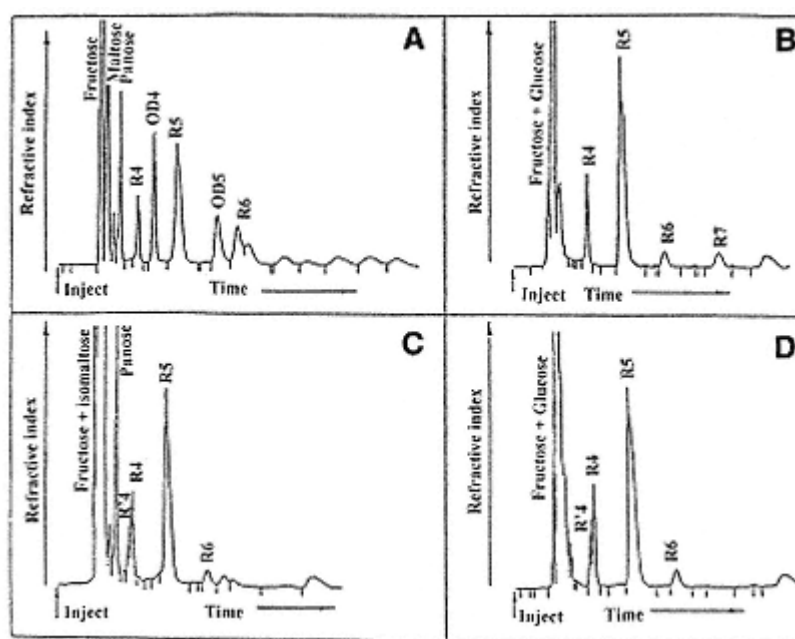


Fig. 3.

HPLC analysis of the GOS synthesized with 100 g/L sucrose, 33 g/L maltose, and 1 U/mL IGT. Products noted ODi represent the oligosaccharides of degree of polymerization i composed of $\alpha(1\rightarrow6)$ linkages and a maltose residue at the reducing end. Products noted Ri represent oligosaccharides bearing an $\alpha(1\rightarrow2)$ linkage. (A) Acceptor reaction products. (B) Acceptor reaction products after glucoamylase hydrolysis. (C) Acceptor reaction products after endo-dextranase hydrolysis. (D) Acceptor reaction products after glucoamylase and endo-dextranase hydrolysis.

As can be seen in **Fig. 3C** and **D**, the ODi GOS can also be eliminated by endodextranase treatment. This treatment enriches the medium mostly with R_4 and R_5 . R_7 can be eliminated by dextranase treatment, but a new $\alpha(1\rightarrow2)$ GOS called R'_4 appears.

17. The equation takes into account the weight of all the monosaccharides involved in the oligosaccharide structure: it comprises: (a) Numerator: all the oligosaccharide concentration (g/L); and (b) Denominator: the entire maltose concentration (g/L), but only the glucosyl moiety (MM = 180 g) of sucrose (MM = 342 g) after elimination of a water molecule ($180 - 18 = 162$ g); that is to say, that only $162/342 = 0.474 \times$ the initial sucrose concentration (g/L) can take part in the oligosaccharide synthesis.

18. Under the test conditions, this yield is about 70%, the fraction of GOS bearing an $\alpha(1\rightarrow2)$ linkage being approx 50%. These results can be modified by changing the reaction conditions, especially the sucrose/maltose (S/M) concentration ratio (Dols et al., in preparation).

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12—

Enzymatic Production of Fructooligosaccharides from Sucrose.

Jong Won Yun and Seung Koo Song

1—

Introduction

Recently many oligosaccharides, such as fructo-, isomalto-, soybean-, and galacto-oligosaccharides, have been widely used in bioindustries as “functional sweeteners” because of their various health-promoting properties, such as being low calorie, and antidental caries, and having bifidogenic functionalities (1–5). Among them, fructooligosaccharides (FOS) from sucrose have received special attention in that mass production is relatively easy, their taste and physiochemical properties are quite similar to those of sucrose, and their functional properties are well elucidated compared with other oligosaccharides (6–8). The important functionalities of FOS are listed in **Table 1**.

FOS have been discovered from many plants, such as asparagus, onion, and Jerusalem artichoke (9–12). However, because production yield of FOS by using these plant-originated enzymes is very low and mass production of enzyme is also quite limited by seasonal conditions, the industrial production of FOS uses mainly several microbial fructosyltransferase (β -D-fructofuranosidase, EC. 2.4.1.7) derived either from *Aspergillus* (13,14) or *Aureobasidium* sp. (15–20).

Depending on the enzyme sources, FOS have different linkages; for instance, fructosyltransferase derived from fungi, such as *Aureobasidium pullulans* and *Aspergillus niger*, produce only 1^F-type FOS with high regiospecificity, whereas plant and several microbial fructosyltransferases simultaneously produce both 1^F- and 6^G-type oligofructosides (21–24). FOS became a common nomenclature only for naming the oligofructosides that are mainly composed of 1-kestose (GF₂), nystose (GF₃), and 1^F-fructofuranosyl nystose (GF₄), in which fructosyl units (F) are bound at the β -2,1 position of sucrose molecule (GF) (**Fig. 1**). Moreover, there exists a slight difference in sugar composi-

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Table 1
Physiological Functionalities of FOS

Prevention of dental caries
 Safe in diabetics
 Proliferation of bifidobacteria and reduction of detrimental bacteria
 Reduction of toxic metabolites and detrimental enzymes
 Prevention of pathogenic and autogenous diarrhea
 Prevention of constipation
 Protection of liver function
 Reduction of serum cholesterol
 Reduction of blood pressure
 Anticancer effect
 Production of nutrients

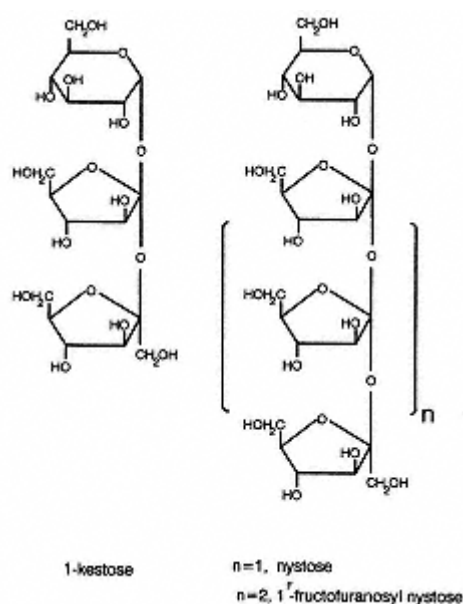


Fig. 1.
Chemical structure for FOS.

tions of FOS by enzyme types and reaction modes, whose individual carbohydrate compositions are listed in **Table 2**.

Current industrial processes for the production of FOS utilize two enzymatic reaction systems using either a batch system with soluble fructosyltransferase (**14**) or a continuous system with immobilized cells entrapped in calcium alginate gel (**16**). Recent processes developed for FOS production that have industrial potential are listed in **Table 3**, and their typical processes are illustrated in

Table 2
Comparison of Compositions (% w/w) of FOS
Produced by Different Enzyme Forms and Reaction Modes

Carbohydrates ^a	Compositions					
	Free enzyme	Immobilized enzyme		Free cells	Immobilized cel	
		Batch	Continuous		Batch	Contir
Glucose (G)	26.1	25.7	27.6	27.7	30.0	26.9
Sucrose (GF)	19.0	19.5	15.7	15.5	15.0	15.1
DP3 (GF ₂)	40.5	39.0	36.4	39.9	26.0	30.9
DP4 (GF ₃)	13.8	14.2	17.5	15.3	19.5	20.1
DP5 (GF ₄)	0.6	1.3	2.7	1.3	7.8	5.6
DP6 (GF ₅)	0	0	0	0	1.7	1.3
Total FOS (GF _n)	54.9	54.5	56.6	56.7	55.0	58.0

^aDP means degree of polymerization; GF₂, 1-kestose; GF₃, nystose; GF₄, 1^F(1-β-fructofuranosyl) nystose; GF₅, 1^F(1-β-fructofuranosyl)₄ sucrose.

Table 3
Current Processes for Fructooligosaccharide Production

Enzyme types, reaction mode ^a	Enzyme source	Productivity, g/L·h ^b	Refs.
IC (semibatch)	<i>A. pullulons</i>	45	(17)
IC (continuous)	<i>A. pullulans</i>	180	(16)
IC (continuous)	<i>Asp. japonicus</i>	234	(28)
IE (continuous)	<i>Aureobasidium sp.</i>	1190	(29)
IE (continuous)	<i>A. pullulans</i>	1174	(30)

^aIC and IE mean immobilized cell and immobilized enzyme, respectively.

^bProductivity was estimated based on the first 30 d of operation of the reactors (total amounts of FOS/U reactor volume/U time).

Fig. 2. Three simple process for FOS production (by use of crude enzyme solution, immobilized cell and immobilized enzyme) from sucrose are described in detail in this chapter.

However, in spite of beneficial health-promoting properties of oligosaccharides, most oligosaccharide syrups that are presently available have an important limitation, i.e., total content of oligosaccharide accounts for at most 60% in total dry weight (16–20). Thus, recent interest in process development for production of oligosaccharides has concentrated on “high-content” commercial product (19). A potential process for high-content FOS syrup using a dual-enzyme system of fructosyltransferase and glucose oxidase (for removal of glucose, which accounts for about 30% in total sugar and acts as a competitive inhibitor during enzymatic reaction in FOS production) is also described.

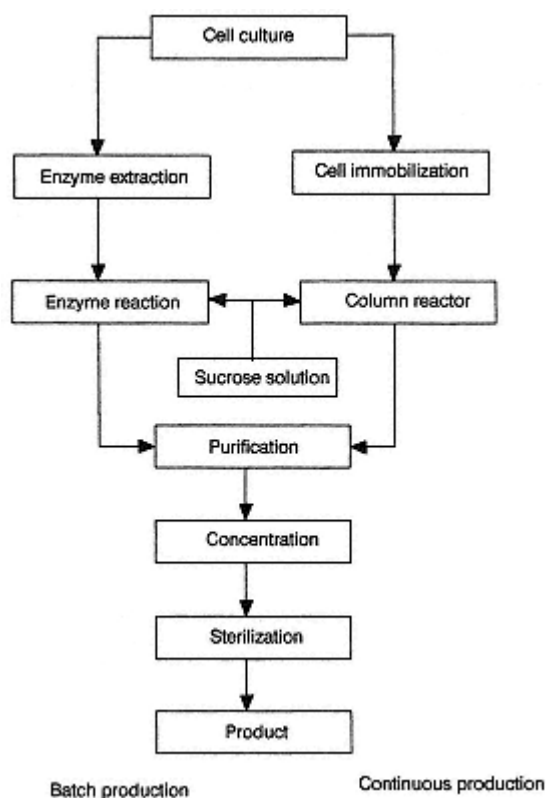


Fig. 2.
Typical processes for FOS production from
sucrose by either crude enzyme or immobilized
cells (enzyme).

2—

Materials

2.1—

Substrates for FOS Production

1. For use in foodstuff ingredient: a commercial food-grade sucrose.
2. For use in feedstock ingredient: raw sugar or sugar molasses (25).
3. For analytical purposes: pure 1-kestose (GF₂), nystose (GF₃), and 1^F-fructofuranosyl nystose (GF₄) (Meiji Seika Kaisha, Ltd., Japan).

2.2—

Enzymes

1. Fructosyltransferase (EC 2.4.1.7): crude enzyme preparation from *A. pullulans*.
2. Glucose oxidase (EC 1.1.3.4): crude enzyme from *A. niger* (Sigma).

2.3—

Immobilization of Whole Cell

A food-grade sodium alginate (e.g., Hayashi Pure Chemical Industries, Ltd., Japan) is needed.

2.4—

Immobilization of Enzyme

1. An ion-exchange resin commercially available (e.g., Diaion HPA series, Samyang Co., Korea) was used for enzyme immobilization.
2. 0.01 M sodium citrate buffer (pH 5.5).

2.5—

Culture Media

Growth medium A consists of 20% sucrose, 2% yeast extract, 0.5% K₂HPO₄, 0.2% MgSO₄·7H₂O, and 1.5% NaNO₃ (see **Note 1**).

3—

Methods

3.1—

Preparation of FOS-Producing Whole Cells.

1. Submerged culture of *A. pullulans* Korean Foundation of Culture Collection (KFCC) 10245 is carried out at 28°C for 36 h in a fermenter containing the growth medium A.
2. Harvest the cells by centrifugation, and wash twice with deionized water prior to immobilization (see **Note 2**).

3.2—

Preparation of FOS-Producing Enzyme

1. Harvest log-phase cells by centrifugation, and resuspend in deionized water to a cell concentration of 20% (w/v).
2. To extract intracellular fructosyltransferase, treat the cell suspension was treated with 2% (w/v) lysozyme (e.g., Kitalase, Kumiai-kagaku, Japan) for 2 h at 45°C, and then filter to produce a clear supernatant.
3. Concentrate the filtrate by dialysis and membrane filtration (mol-wt cutoff 10,000 Dalton, e.g. Amicon YM10 membrane).
4. The resulting enzyme solution is used as crude enzyme without further purification.

3.3—

Batch Production of FOS

1. Five units per gram sucrose are used in sucrose solution ranging from 700 to 850 g/L (see **Subheading 3.8.**).
2. The reaction is carried out at 55°C for 20–25 h with gentle agitation.

3.4—

Whole-Cell Immobilization for Continuous Production of FOS

1. Mix the cell suspension containing 20% (w/v) of wet cells thoroughly with 3% (w/v) sodium alginate solution (previously prepared by heating 60–70°C) in the volume ratio of 1:2 (see **Note 3**).
2. Extrude the mixture through syringe needles (gage no. 25) to form small beads of about 2 mm diameter, dropping into 0.1 M calcium chloride solution (see **Note 4**).
3. Cure the beads for 2 h at a room temperature, and harden overnight at 4°C in calcium chloride solution.
4. After washing thoroughly, saturate the resulting immobilized cells with 800–900 g/L sucrose solution prior to usage (see **Note 5**).

3.5—***Immobilization of Crude Fructosyltransferase***

1. A high porous ion-exchange resin (e.g., Diaion HPA25, Samyang Co., Korea) is equilibrated with 0.01 M sodium citrate buffer (pH 5.5) for 24 h, and packed into a glass column with a suitable bed volume.
2. Prepare a crude enzyme solution in 0.01 M citrate buffer, pH 5.5.
3. Pass the resulting enzyme solution into the column at an appropriate flow rate (e.g., superficial space velocity 0.5–1 h⁻¹, i.e., 0.5-L bed volumes of substrate/h; see **Note 6**).
4. After immobilization for 10 h at an ambient temperature, washing should be continued for at least five bed volumes.
5. No activation of support was carried out before or after immobilization in order to simplify the immobilization procedure and to guarantee the quality of product.

3.6—***Continuous Production of FOS Using Immobilized Cell or Enzyme Reactor***

1. A concentrated sucrose solution (e.g., 850 g/L) is fed upward continuously through to the reactor in which the temperature is kept constant (e.g., 50°C) by circulating hot water through the jacket (see **Notes 7–9**).
2. Maintain the feed flow rate around SV 0.15–0.2 h⁻¹ for immobilized cell reactor and SV 1.5–2.5 h⁻¹ for immobilized enzyme reactor (see **Note 10**).
3. Continuous operation is carried out for 100 d with immobilized cells or 30 d with immobilized enzyme (see **Notes 11** and **12**).

3.7—***Production of High-Content FOS***

1. A mixed-enzyme system of fructosyltransferase (10 U/g substrate) and glucose oxidase (15 U/g substrate) is employed in a 2-L stirred-tank reactor containing 0.7 L of 400 g/L sucrose solution (see **Note 13**).
2. The mixed-enzyme reaction is carried out under the following reaction conditions: pH, 5.5 continuously controlled using 1 M NH₄OH; temperature, 40°C; agitation speed, 550 rpm; oxygen flow rate, 0.7 L/min; reaction time 25 h (see **Notes 14** and **15**).
3. The activity of glucose oxidase is evaluated from the disappearance rate of glucose in a 2-L stirred-tank reactor under the following assay conditions: substrate, 0.7 L of 400 g/L β-D-glucose; oxygen flow rate, 0.7 L/min; agitation speed 550 rpm; 1 mL of glucose oxidase solution prepared in 0.1 M citrate buffer; temperature 40 °C, pH 5.1.
4. One unit of glucose oxidase is defined as enzyme activity required to oxidize 1 μmol of glucose at the conditions described above. Determine the amounts of glucose by the glucose oxidase-peroxidase method (Sigma diagnostics kit No. 510-A).

3.8—***Enzyme Assay***

1. The fructosyltransferase activity of free cells and soluble enzyme are determined by measuring the released glucose using the glucose oxidase-peoxidase method (Sigma).

2. One enzyme unit is defined as the amount of enzyme activity required to produce 1 μmol of glucose/min under the following conditions: 770 g/L sucrose 7.5 mL, enzyme solution 0.2 mL, 0.1 M citrate buffer, pH 5.5, 2.3 mL, 55°C, 1 h.
3. Enzyme activity is determined after terminating the reaction by boiling the mixture in a water bath for 15 min.
4. In the case of immobilized enzyme and cells, immobilized cells or enzyme are filtered using a Whitman filter paper no. 9.
5. React 1 g of the immobilized enzyme or cells (wet wt) with the same sucrose solution and buffer system as described in **step 2**.

3.9—

Analytical Methods

1. A direct method of measuring FOS is possible by HPLC. FOS and free sugars are easily separated on an ion-exchange column (e.g., HPX-87C and HPX-42C, Bio-Rad) connected to a refractive index detector.
2. The column temperature is kept constant at 85°C.
3. Use water as mobile phase at a flow rate of 0.6 mL/min.
4. Total amount of FOS is determined by the sum of 1-kestose (GF_2 : G, glucose; F fructose), nystose (GF_3), 1^F-fructofuranosyl nystose (GF_4), and 1^F-(fructofuranosyl)₄ sucrose (GF_5).
5. Paper and thin-layer chromatographic analysis also can be used for identification of degree of polymerization (DP) of oligosaccharides (see **Note 16**).

4—

Notes.

1. Although fermentation parameters of aeration, agitation, pH, and temperature should be established for each microorganism, the general conditions for producing fructosyltransferase by growing cultures of organisms are well established. For example, sucrose is the best carbon source for both cell growth and enzyme activity, maintaining pH above 5.5 is important, and the optimum temperature for growth is ~30°C. Intracellular fructosyltransferase activity can be greatly enhanced by increasing the amount of Mg ion concentration in culture medium (e.g., 0.5 M).
2. Cells are easily harvested by a basket-type centrifuge on a large scale.
3. Alginic acid, a copolymer of β -D-mannuronic acid and α -L-guluronic acid linked by (1→4)-glucosidic linkages, has been most widely used for entrapment of microbial cells (**26**). Although the majority of laboratory chemical suppliers offer various grades of alginates, in this laboratory for FOS production, the sodium alginate supplied by Hayashi Pure Chemicals Ltd. has proven most satisfactory for entrapment of *A. pullulans* (**16,17**).

4. For small-scale bead formation, the suspension of cells in sodium alginate is passed dropwise into a solution of calcium salt (0.08–1 *M*) using a syringe with a suitable diameter. For industrial-scale production of beads, several techniques can be taken into consideration. Multichannel pump or multinozzle devices can be used (27). As depicted in **Fig. 3**, in this laboratory, a schematic apparatus for mass bead formation consisting of two vessels has been developed (main vessel ca.

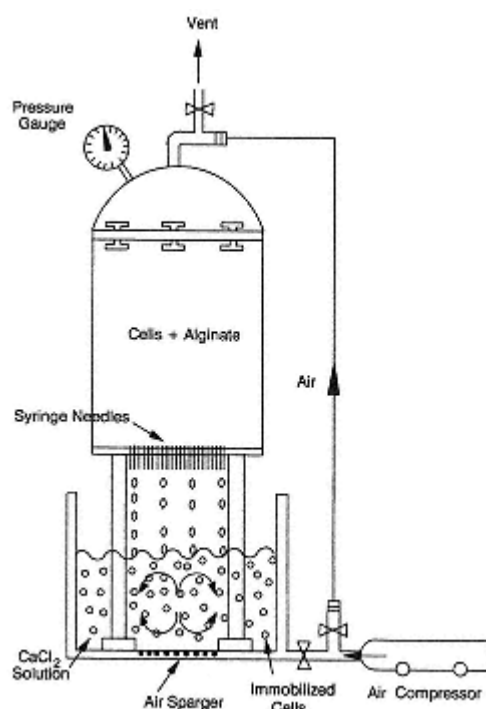


Fig. 3.
A schematic diagram of an apparatus for production
of immobilized cells.

100 L contains a cell-alginate mixture, outside vessel ca. 500 L contains calcium chloride solution) whose porous bottom plate was installed with 500 syringe needles. The air supply device is designed for both extrusion of the cell-alginate mixture and for gentle stirring of the calcium chloride solution to eliminate the possibility of bead coalescing. First the cell suspensions were well mixed with sodium alginate and poured into the main vessel. After closing the lid of the vessel tightly, bead formation is started with supply of air (produced by an air compressor) through the center hole in the lid of vessel. With 500 needles installed, about 100 L of beads can be made/only 1 h.

5. When the cell-alginate mixture is extruded through syringe needles of gage no. 25, small beads in diameter of about 2 mm are usually made. These small beads do not have the satisfactory strength to overcome deformation from spheres to flattened dodecahedrons. Thus, it is submerging the beads into sucrose solutions required (preferably in 500–800 g/L) overnight at a refrigerated temperature prior to packing the beads into enzyme reactor.

6. Partial purification of the enzyme by a conventional purification method gives rise to enhanced immobilization yield and operational stability.

7. The pH of feed solution (naturally maintained at about 6.0–7.0) remains essentially constant throughout the operation. Normally an introduction period of

20–24 h is required to reach a steady state. Therefore, all the samples are taken after 24 h of operation.

8. To avoid a channeling problem in a real reactor operation (owing to a density difference between water and sucrose solution), 300 mL of sucrose solutions ranging from 10 to 50% (w/v) are run consecutively. Operation of the column is facilitated by the upward flow of substrate such that self-compression of the immobilized enzyme causing a clogging problem is minimized. Long-term stability of the immobilized enzyme is evaluated using 600 g/L sucrose at a flow rate of 130 cm³/h (SV 2.7 h⁻¹) at 50°C. Unbuffered sucrose should be used throughout the experiments.

9. It is very important to keep the reaction temperature at 50°C, because the longterm stability of the immobilized cells or enzyme is significantly sensitive to temperature. For instance, the immobilized cells maintain their initial enzyme stability over 100 d at 50°C, but only 35 d at 55°C.

10. Although high concentrations of sucrose solution (up to 750 g/L) may be used as a substrate for both batch and continuous process of FOS production, in a column reactor operation packed with either immobilized cells or immobilized enzyme, a slightly lower concentration (e.g., 700 g/L) is recommended owing to difficulties in feeding because the viscosity of the solution is very high.

11. Immobilized enzyme columns are essentially superior to immobilized cell columns from a practical point of view, i.e., the immobilization method is rather simple, and unit volumetric productivity is greater. However, the operational stability of the immobilized cells (stable for 100 d) is higher than that of immobilized enzyme (stable for 35 d) (**16,28**). It should be stressed that both productivity and operational stability should be simultaneously considered in determining the industrial feasibility of FOS production.

12. From the practical standpoint that batch production with the extracted enzyme needs an additional process for the removal of residual enzyme contained in the reaction products, a continuous process with immobilized enzyme or cells is more favorable.

13. When the mixed-enzyme system of fructosyltransferase and glucose oxidase is employed for high-content FOS production, a significant difference appears in optimum temperature and sucrose concentration for the enzyme activities. The optimum temperature for the glucose oxidase and the fructosyltransferase are 35 and 55°C, respectively. For the glucose oxidase, a rise in temperature means a decrease in oxygen concentration. Oxygen solubility essentially decreases on temperature rise and tends to offset the benefit of the temperature rise in enzyme activity. Considering both the maximum FOS productivity and the minimum resistance of oxygen transfer, the best sucrose concentration is below 400 g/L.

14. When glucose oxidase converts glucose to gluconic acid, the reaction pH decrease rapidly. Thus automatic pH control is required.

15. NH₄OH used for pH control is completely removed during evaporation process in a polishing step.

16. For identifying the degree of polymerization of the products, thin-layer chromatographic analysis is carried out on silica gel 60 plates. The plates are developed with a solvent system of isopropanol-2-ol/ethyl acetate/water (6/2/2 by vol). Sugars

are visualized by spraying the plates with sulfuric acid/phenol reagent and heating 120°C for 5 min.

17. Generally, the final FOS syrup commercially available has the concentration of over 800 g/L: as a polishing step, concentration of the reaction products is performed by a conventional evaporation process.

18. In the sterilization process, although either heat or UV sterilization methods can be recommended, the UV method is favored, because color may be produced by heat sterilization at high sterilization temperatures, and continuous sterilization is also possible with UV sterilization.

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13—

Enzymatic Production of Inulooligosaccharides from Inulin

Jong Won Yun and Dong Hyun Kim

1—

Introduction

Inulin is a fructose polymer found in plants such as Jerusalem artichoke, chicory, and dahlia (**1–2**). It has been widely investigated as a source for the production of ultra-high-fructose syrup through enzymatic hydrolysis by exoinulinase (EC 3.2.1.80; β -D-fructan fructohydrolase) acting alone or synergically with endoinulinase (EC 3.2.1.7; 2,1- β -D-fructan fructanohydrolase) (**3–7**).

Oligosaccharides such as fructo-, isomalto-, soybean-, and galactooligosaccharides are one of the most popular functional food ingredients because of their excellent functionalities (**8–12**). There is an important limitation, however, to the wide application of the oligosaccharides in spite of their beneficial health-promoting properties: oligosaccharide content accounts for only 50–60% on a total sugar basis (**13–16**). Thus, recent interest in process development for the production of oligosaccharides has concentrated on high-content commercial product (**17–19**). In this regard, inulin can be a promising source for oligosaccharide production provided that strong endoinulinase acts on inulin in the absence of exoinulinase or invertase activities.

Inulooligosaccharides (IOS) from inulin have very similar chemical structure to fructooligosaccharides from sucrose whose functionalities have already been extensively characterized (**8,9,11**). In addition, they are presently regarded as a soluble dietary fiber (**20**). Although it has been reported that IOS show quite similar functional properties to fructooligosaccharides, and a few researchers have described their potential for alternative oligosaccharides (**21**), extensive experimental results are scarcely presented. The authors recently reported a process for the production of IOS from inulin with high yield using a crude endoinulinase preparation derived from a new isolated *Pseudomonas* sp. (**22–25**).

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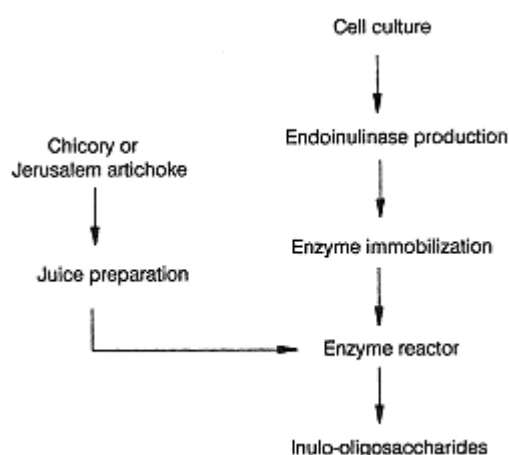


Fig. 1.
A proposed process for the inulooligosaccharide production from inulin-rich raw material.

Economic considerations dictate that, if used, inulinase would be best utilized in an immobilized form. However, immobilization of inulinase activity is also scarcely documented in the literature compared with other enzyme systems involved in sugar bioconversion. Several gel matrices, including calcium alginate, agar, and gelatin together with support materials such as a cellulose derivative and chitin, have been examined for inulinase immobilization (26–31). However, the object of these immobilization systems was not to produce IOS but to produce high-fructose syrup. A proposed process for IOS production on an industrial scale is illustrated in **Fig. 1**. It has been described that no serious additional problems occurred between pure inulin and inulin-rich raw materials such as chicory and Jerusalem artichoke for the enzymatic production of high-fructose syrup (32,33). Therefore, although pure inulin was used as substrate, the protocols described here would be successfully applied to IOS production from such raw materials.

Two protocols for IOS production are given detailing the procedures: (1) with crude enzyme preparations, from a *Pseudomonas* sp., and (2) with its immobilized enzyme.

2—

Materials

2.1—

Materials

1. Pure nonhydrolyzed inulin derived from dahlia tubers (Sigma, St. Louis, MO) which has a mean degree of polymerization (DP) of ca. 35, was used as a model substrate.

2. 0.05 M Sodium acetate buffer.

3—

Methods

3.1—

Preparation of Crude Endoinulinase Solution

1. *Pseudomonas* sp., a new isolate obtained in our laboratory, was cultivated at 45°C for 60 h in a 250 mL flask containing 50 mL of medium composed of 1% (w/v) inulin, 0.8% (NH₄)₂HPO₄, 1.5% corn steep liquor, 0.05% KCl, 0.05% MgSO₄ · 7H₂O, and 0.003% FeSO₄ · 7H₂O (see **Note 1**).
2. After removal of the cells by centrifugation (10,000g), the supernatant containing extracellular endoinulinase was concentrated by dialysis and membrane filtration (mol-wt cut off 30 kDa; Microconcentrator, W.R. Grace & Co., Beverly, MA).
3. The resulting crude enzyme solution was used throughout the experiments without further purification (see **Note 2**).

3.2—

Endoinulinase Immobilization

1. An anionic ion exchange resin, Diaion WA30 (Samyang, Seoul, Korea) was equilibrated with 0.01 M sodium acetate buffer, pH 4.5, for 24 h, and packed into a glass column (1.2 × 30 cm) with a bed volume of ca. 30 mL (see **Note 3**).
2. The crude enzyme solution was prepared in 0.01 M sodium acetate buffer (pH 4.5) and then the resulting enzyme solution was passed into the column at a flow rate of 30 mL/h (see **Notes 4** and **5**).
3. After immobilization for 10 h at an ambient temperature, the column was washed thoroughly using 500 mL of deionized water.

3.3—

Batch Production of Inulooligosaccharides (see Notes 4 and 5).

1. Batch enzyme reactions were carried out in 250 mL stoppered flasks containing 50 mL inulin solution in a rotary shaker incubator at 55°C and 150 rpm. Enzyme concentration for IOS production was 15 U for soluble enzyme and 25 U/gram inulin, respectively.
2. Samples were taken at regular intervals and the reactions were stopped by heating the reaction mixtures in a boiling water bath for 10 min.

3.4—

Determination of the Most Suitable Reaction Time in Batchwise Production of IOS from Inulin (see Notes 10–12)

1. To obtain basic kinetic data, enzyme reactions were carried out initially with various dosages of enzyme with 4 substrate concentrations ranging from 50–200 g/L inulin. The maximum yields of oligosaccharides achieved were similar irrespective of initial concentration of enzyme or substrate, except that the reaction time was reduced as the enzyme dosage and inulin concentration increased.

2. Using the kinetic data, the relationships between initial enzyme concentration employed and reaction time to reach yield maxima were derived. Plots of the logarithm of enzyme dosage (E_0) against logarithm of the time (t_M) yield straight lines of the same slopes (-1.025), giving the equation, $\log(t_M) = -1.025 \log(E_0) + b$, where b varies with initial concentration of inulin (I_0) and can be determined by the time intercept of each line.

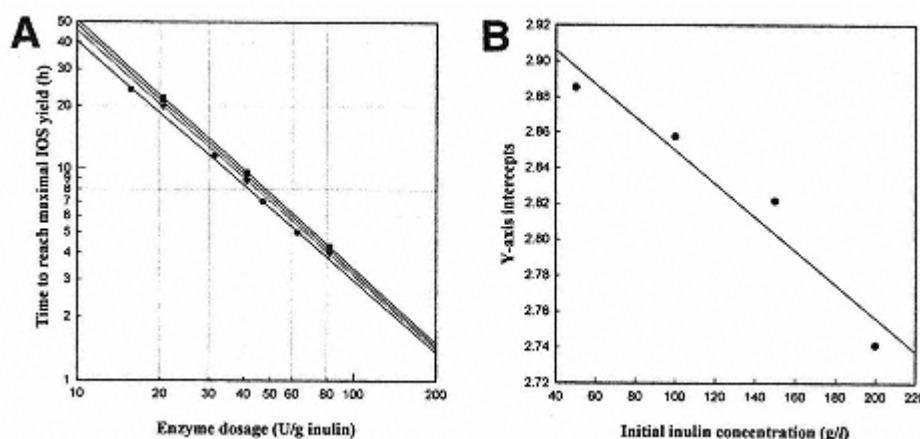


Fig. 2.

Relationships between enzyme dosage and time to reach maximum inulooligosaccharide yield (A); initial inulin concentration and intercept of time to reach yield maximum (B). Inulin concentration as g inulin/L: (j) 50; (m) 100; (.) 150; (d) 200.

3. Finally this relationship may be incorporated into one kinetic description to get a view of the whole reaction performance. Thus, a plot of $\log(I_0)$ against $\log(b)$ was constructed, which also yielded a straight line of slope, -0.001. Consequently, the reaction time to reach maximum yield may then be determined from the following equation: $\log(t_M) = -1.161 \log(E_0) - 0.001 I_0 + 2.94$ (Fig. 2) (see Note 13).

3.5—

Reactor Operation for Continuous Production of Inulooligosaccharides

1. To prevent a channeling problem in actual column operation (due to a density difference between water and inulin solution), 150 mL of inulin solutions ranging from 5 to 50 g/L were run consecutively. To evaluate a reactor performance, the column was operated continuously at various flow rates using 50 and 100 g/L of inulin as substrate at 55°C, respectively (see Note 14).
2. Operation of the column was facilitated by the upward flow of substrate such that self-compression of the immobilized enzyme causing a clogging problem was minimized.
3. The effluent concentration of inulooligosaccharides was analyzed after 24 h operation, when a steady state was essentially established.
4. Long-term stability of the immobilized enzyme was evaluated using 50 g/L inulin at a flow rate of 12 mL/h (Superficial Velocity, SV 1.1 h⁻¹) at 55°C (see Notes 15–19).

3.6—

Enzyme Assay

1. For soluble endoinulinase, activity was assayed by incubating 2 mL enzyme solution with 2% (w/v) inulin prepared in 0.01 M sodium acetate buffer, pH 5.5, at 55°C for 60 min.

2. In the case of immobilized enzyme, the enzyme solution was replaced by 1 g of immobilized enzyme (wet weight after filtration).

3. One enzyme unit was defined as the amount of enzyme responsible for the production of 1 μ M inulobiose (mol wt 342) per min under the conditions described above (see **Note 20**).

3.7—

Analytical Methods

1. Carbohydrates are directly analyzed by an HPLC system using an Aminex HPX-42C column (0.78 $\times$ 30 cm, Bio-Rad, CA) and a refractive index detector. The column temperature was kept constant at 85°C and water is used as the mobile phase at a flow rate of 0.6 mL/min.

2. The total inulooligosaccharides are estimated as the sum of inulobiose (F_2) and other oligofructosides ($GF_n + F_n$; mainly of F_n) with DPs of ranging from 3–7.

3. To determine the DPs of the products, thin layer chromatographic analysis is carried out on precoated silica gel plates (Whatman K5). The plates are developed with a solvent system of acetonitrile/ethyl acetate/*n*-propanol/water (85/20/50/50, by volume).

4. The sugar spots were visualized by spraying the plates with 5% (w/v) sulfuric acid solution containing 0.5% (w/v) α -naphthol and heating at 120°C for 10 min.

4—

Notes

1. Sucrose and extracts of chicory or Jerusalem artichoke may also be used as useful carbon source for endoinulinase production from *Pseudomonas* sp.

2. The endoinulinase preparation can be utilized for IOS production, achieving a satisfactory IOS yield (max. 83%). Thus, it is not a prerequisite to purify the endoinulinase for IOS production.

3. Various kinds of ion exchangers, which are inexpensive and commercially available, can be used successfully for endoinulinase immobilization. An anionic polystyrene-based ion exchanger, Diaion WA30, was the most appropriate carrier for enzyme immobilization due to its excellent adsorption yield (about 60%).

4. Continuous immobilization is essentially superior to batch adsorption in immobilization yield by overcoming adsorption equilibrium.

5. No activation of support is recommended before or after immobilization in order to simplify the immobilization procedure and to guarantee the quality of product.

6. The maximum yield (75.6%) in total inulooligosaccharides was observed when 50 g/L inulin was utilized by soluble endoinulinase within 25 h. It was found that the products consist of inulooligosaccharides ranging from DP2 to DP7, being mainly DP2 and DP3 by thin layer chromatographic analysis. Moreover, considerable amounts of fructose and glucose and trace quantity of sucrose, regarded as by-products, were also released. **Figure 3A** shows a typical reaction pattern of soluble endoinulinase for IOS production using 50 g/L inulin as a substrate. Inulin was completely hydrolyzed leading to successive formation of DP2, DP3, and other higher DP oligosaccharides.

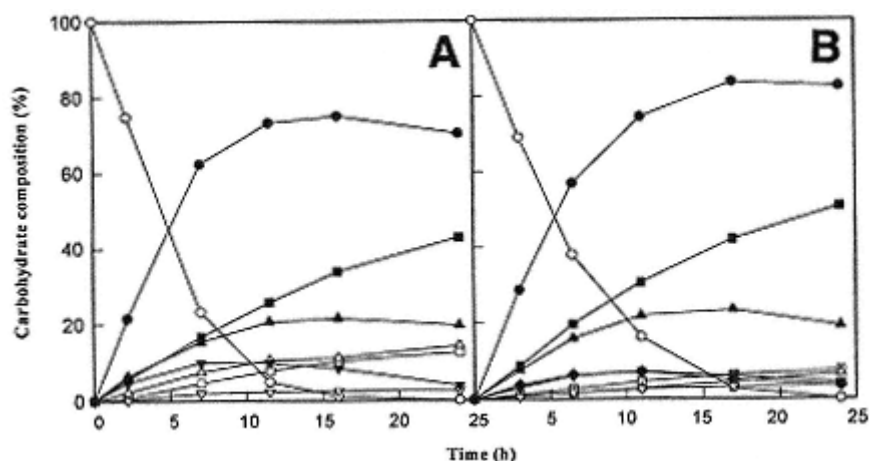


Fig. 3.

Typical time-courses of various sugar components in batchwise production of inulooligosaccharide production from inulin using soluble (A) and immobilized endoinulinase (B) with 50 g/L inulin: (s) inulin; (d) total inulooligosaccharides; (j) DP2; (m) DP3; (.) DP4; (n) fructose; (h) glucose; (.) sucrose.

7. It should be noted here that the concentration of DP2 was continuously increasing toward the end of the reaction, whereas higher oligosaccharides, namely DP3, DP4 and other products of higher DP, were gradually hydrolyzed after reaching their maxima.

8. On the basis of data from substrate specificity, i.e., endo-inulinase used in this work cannot hydrolyze sucrose (GF), 1-kestose (GF₂), or nystose (GF₃) but acts on 1^F-fructofuranosyl nystose (GF₄), it is likely that early product F₄ is hydrolyzed to F₃ and F, GF₄ to GF and F₃, F₅ to F₃ and F₂.

9. Considering that more rapid decrease occurs in the concentration of the higher DP oligosaccharides as the reaction proceeds, it appears that the enzyme preferentially acts on the higher oligosaccharides than on small oligomers.

10. In most enzymatic degradation reactions of high molecular biomolecules, such as starch and inulin, excess of reaction is frequently unfavorable when oligomers are more desired products rather than monomers, which are completely degraded products (34). Thus, termination of the batchwise enzyme reaction within the most suitable reaction time should be considered. Therefore, it is necessary to predict an accurate reaction time to reach the maximum yield of inulooligosaccharide from inulin because most of the oligosaccharides produced are further degraded by successive actions with endoinulinase.

11. Crude endoinulinase from *Pseudomonas* sp. possesses the capability of producing a considerable amount of free fructose as a byproduct during inulooligosaccharide production from inulin. Consequently, the yields of inulooligosaccharides gradually decrease after reaching their maxima due to further hydrolysis of oligosaccharides to fructose.

12. These kinetic descriptions of enzyme reaction enable the prediction of performance for batch production of inulooligosaccharides from inulin. Moreover, this technique may be widely applied for other systems in enzymatic hydrolysis reactions of high molecular biomolecules where intermediates are target products like oligosaccharides.

13. Although the kinetic description can vary by enzyme source employed, it appears that there is no significant difference; for example, a purified endoinulinase from *Aspergillus ficuum* (a commercial inulinase preparation, Novozyme 230, Novo A/S) gives a quite similar equation, i.e., $\log(t_M) = -1.025 \log(E_0) - 0.001(I_0) + 2.65$.

14. Common operating variables affecting the performance of the immobilized enzyme column are the flow rate of feed, feed concentration, and operation temperature (35). Although the optimal pH for activity of immobilized enzyme is 4.0–5.0, buffering the inulin solution should be avoided from the practical point of view.

15. In general, the residence time (1/SV) is calculated on the basis of void volume of the reactor (void fraction of the enzyme reactor was 0.36). Unbuffered inulin solutions without sterilization should be used throughout the experiments for the sake of practical application of the system.

16. In long-term operation of the enzyme reactor, operation temperature is a very important factor in maintaining initial enzyme activity for a prolonged reaction time. It has often been suggested that although the optimum temperature for the activity of the immobilized enzyme was high, it would be better to operate the column at a reduced temperature to prevent activity loss during long periods of operation. However, in this system it is impossible to run the reactor at a reduced temperature below 55°C due to poor oligosaccharide yield (for instance, 63% at 50°C).

17. To achieve more than 80% conversion based upon total solid content as well as to maximize the productivity, the column should be operated at flow rate below SV 1.1 h⁻¹ and feed concentration of 50 g/L, respectively. The results suggest that higher inulin concentrations up to 50 g/L might pose a certain degree of diffusional problem within the immobilized enzyme reactor. Moreover, pure inulin precipitates easily in solutions of 10% or more upon cooling to room temperature.

18. The immobilized enzyme reactor can be run successfully for 15 d achieving an oligosaccharide yield of 83% without any significant loss of initial enzyme activity. Thereafter the enzyme activity was gradually decreased. The half-life of the immobilized enzyme was 35 d. Although no hardening agent was not used for enzyme immobilization, the operational stability achieved was relatively high compared with those of immobilized inulinase systems documented in the literature (26–31).

19. In contrast with soluble enzyme, the immobilized enzyme (83%) interestingly increased the total IOS yield compared with the soluble enzyme (76%). This is probably because the enzyme immobilized on an internal surface of the ion exchanger, causing a certain degree of diffusional restriction of high molecular substrates and products. Thus, actual contact time between enzyme and sugar molecules at a given superficial reaction time was essentially prolonged, allowing the enzyme to catalyze further hydrolysis of higher oligomers into inulobiose without attack on inulobiose itself. This result can be supported by the fact that

Table 1
Composition of Inulooligosaccharides Produced from Different Initial Concentrations (g/L) of Inulin Using Solu Endoinulinase

	Composition (% w/w) ^a		
	50	100	150
Carbohydrates			
Inulin	4.0	4.5	9.0
Glucose	8.5	8.2	7.9
Fructose	9.5	9.7	11.3
Inulooligosaccharides			
DP2	29.8	28.7	25.1
DP3	21.4	21.4	19.8
DP4	8.1	7.9	7.8
>DP4	16.3	17.1	16.9
Total oligosaccharides	75.6	75.1	69.6

^aProduct compositions are those at the reaction time when oligosaccharide formation was maximum.

Table 2
Composition of Inulooligosaccharides Produced from Different Initial Concentrations of Inulin (g/L) by Immobilized Endoinulinase

	Composition (% w/w) ^a		
	50	100	150
Carbohydrates			
Inulin	2.6	1.7	1.7
Glucose	6.4	6.5	6.3
Fructose	4.4	4.6	4.4
Sucrose	3.1	3.4	3.7
Inulooligosaccharides			
DP2	41.8	42.0	40.5
DP3	23.2	23.8	24.2
DP4	6.0	6.1	6.5
>DP4	12.5	11.9	12.7
Total oligosaccharides	83.5	83.8	83.9

^aProduct compositions are those at the reaction time when oligosaccharide formation was maximum.

endoinulinase in this work has higher specificity on the sugar molecules which have high DP. The critical difference in sugar compositions between soluble and immobilized enzyme (see **Tables**). The high contents of inulobiose in the IOS products catalyzed by immobilized endoinulinase were

be a favorable property because bifidogenic functionality of the product with lower DP is superior to those of higher oligosaccharides (27).

20. The 3,5-dinitrosalicylic acid method widely used for determination of reducing sugars is not a suitable tool in the evaluation of endoinulinase activity for IOS production because inulin and its hydrolyzates were significantly hydrolyzed during assay condition. Thus, those degradation products from inulin, such as sucrose, glucose, and fructose, should be excluded for activity evaluation (36).

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14—

One-Pot Enzymatic Synthesis of Sialyl T-Epitope

Vladimir Kren *

1—

Introduction

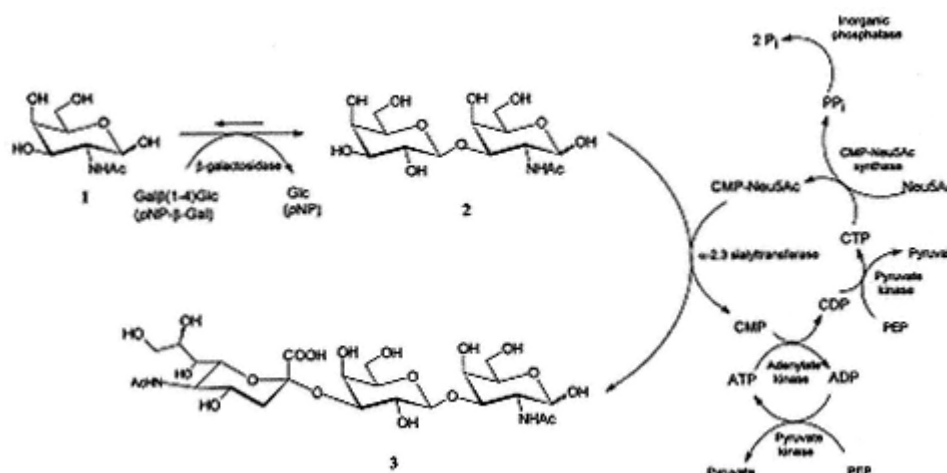
This chapter demonstrates a practical approach to a rather sophisticated multienzymatic one-pot reaction in glycoscience. The first applications using the multienzyme system in glycosylation were mostly targeted to generation or regeneration of the activated glycosyl donors *in situ* (kinases, phosphorylation sequences).

Recently, more sophisticated approaches emerged, e.g., sequential use of more glycosyltransferases, including cofactor regeneration (1–4), or even sequential use of glycosidases and glycosyltransferases (5,6). These approaches sometimes tend to simulate real enzymatic sequences working *in vivo*.

Multienzyme systems are applied mainly to the preparation of complex sialooligosaccharides. Sialic acid (Neu5Ac) is a common structure in glycoconjugates, and sialylated structures are involved in a variety of biological processes. In addition to their role in secretion, immunogenicity, circulation half-life of glycoproteins, and cellular recognition phenomena, sialylated structures form crucial structural elements of some antigenic determinants that have been identified as tumor markers.

Thomsen-Friedenreich (*T*) antigen, (Gal β [1→3]GalNAc α -O-Ser), belongs to the blood-group-related antigens expressed in epithelial tumors (7). A sialylated epitope (Neu5Ac α [2→3]Gal β [1→3]GalNAc [3]) (Scheme 1) can be found in GM₁ glycolipid, human erythrocytes (8), and in a bone marrow macrophage lectin (8,9). Hemolytic-uremic (H-U) syndrome with suckling infants is caused by a microbial toxin (enzyme) neuraminidase. It causes primarily desialylation of sialyl-*T*-antigen in erythrocytes followed by hemolysis and further pathological effects. A diagnostic assay of early stages of this syn-

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Scheme 1.
One-pot multienzymatic synthesis of sialyl T-epitope.

drome is based on the radioimmunoassay with monoclonal antibodies (MAbs). T-antigen (epitope), sialyl-T-antigen (epitope), and their derivatives are required for this assay and for possible immunotreatment of H-U syndrome. This epitope is of considerable importance for immunological studies and, e.g., for the preparation of synthetic antitumor vaccines (10,11).

Enzymatic approaches to the synthesis of these epitope structures and their derivatives proved to be useful (12–14), in particular those based on the glycosyltransferases coupled with *in situ* regeneration of sugar nucleosides (1,4).

The starting material for the preparation of this sialyl trisaccharide (3) is Gal β (1 $\rightarrow$ 3)GalNAc (2), which could be obtained either by a complicated multistep synthesis (15) or by an enzymatic transglycosylation employing sequential use of β -galactosidases from bovine testes (synthesis) and *Escherichia coli* (hydrolysis of side products) (16). Both methods and the respective separation procedures are rather complicated, and this is reflected in an extremely high price of the commercial product (approx \$70/1 mg).

1.2—

Principle of the Method.

A combined sequential use of glycosidase together with glycosyltransferase, including a cofactor regeneration, transforms complicated multistep reactions into a one-pot reaction (5), thus avoiding laborious purifications of the intermediates. This reduces considerably the costs of the procedure, using a cheaper glycosidase, and a cheap substrate as well. Reversed hydrolysis of the disaccharide intermediate is blocked by its further glycosyltransferase-mediated sialylation, thus forming an oligosaccharide that is no longer a substrate for the glycosidase (Scheme 1).

The integral part of the system is a CMP-Neu5Ac generating system that involves CMP-Neu5Ac synthase and CTP regenerating cycle based on kinases (**2**). Energy is supplied by phosphoenolpyruvate. An inorganic phosphatase (pyrophosphatase) is used to remove feedback inhibition of CMP-Neu5Ac synthase caused by pyrophosphate.

In this multienzyme reaction, a rather complicated problem had to be solved, i.e., to bring together the quite distant pH optima of the enzymes used.

The pH optimum of CMP-Neu5Ac synthase (calf brain) is 9.0 (**17**), that of α -2,3 sialyltransferase (pork liver) (α -2,3 ST) 6.5 (**18**) and that of β -galactosidase from bovine testes (BTG) 4.3 (**19**). Although the pH optimum of α -2,3 ST is 6.5, the preparative reactions are usually performed at pH 7.5 to prevent decomposition of CMP-Neu5Ac at lower pH. This pH is still suitable for CMP-Neu5Ac synthase and for other enzymes required for CTP regeneration.

The crucial question was whether BTG would be able to synthesise the intermediate disaccharide (**2**) at a pH quite distant from its pH optimum.

According to Distler and Jourdian (**19**), the activity of BTG at pH 7.5 (substrate lactose) is approx 19% of its maximum activity. When *p*-nitrophenyl β -D-galactopyranoside (*p*-NP-Gal) is used, the rate of galactose release can be increased to approx 30% owing to its lower K_m . The synthetic transglycosylation activity of glycosidases is quite often pronounced at alkaline conditions than at the pH optimum (**20**). Therefore, such a shift should support the transglycosylation reaction. Further on, the activity of BTG is about two times higher in the presence of *N*-acetylgalactosamine (**19**), which obviously serves as an acceptor for galactose released, thus shifting the equilibrium of the reaction. This phenomenon was of advantage as well.

We tested these hypotheses using the real reaction milieu (Na-cacodylate 25 mM, MnCl₂ 20 mM, NaCl 75 mM, Triton X-100 1.4% w/v), and *p*-NP-Gal as a substrate. The BTG activity at pH 6.5, 7.0, and 7.5 compared to that at pH 4.3 was 42, 30, and 26%, respectively. GalNAc in the concentration 250 mM elevated the activity 3.6 times at pH 7.5. TLC (*n*-propanol/NH₃ 1 M/H₂O 6:2:1) showed the formation of the intermediate **2** in the reaction mixture within 1 h.

To check whether the reaction of α -2,3 ST can proceed in the presence of *p*-nitrophenol released from *p*-NP-Gal, and to test the coupling of transgalactosylation of GalNAc with the transfer of Neu5Ac to the acceptor disaccharide, we used radioactive labeled CMP-Neu5Ac. When lactose was used as a galactosedonor instead of *p*-NP-Gal, the yield of the product **3** was 20% higher. However, it was decided to use *p*-NP-Gal for preparative purposes to simplify the purification procedure (eliminating the formation of trisaccharides from lactose). This part of the reaction sequence without CMP-Neu5Ac regeneration was optimized using the radioactive-labeled substrate. It was shown that the BTG preparation used did not hydrolyse CMP-Neu5Ac.

This part of the reaction sequence was coupled to a modified multienzymatic system regenerating CMP-Neu5Ac (**2,4**). The final composition of the reaction mixture on a semipreparative scale is given in **Subheading 3.3**.

The reaction can be monitored by the colorimetric system that enabled the determination of the proportion of free Neu5Ac, CMP-Neu5Ac, and the product (**3**) at any time, and therefore, to optimize the reaction course and reactant additions (**16**). However, monitoring of the intermediates is not crucial. Formation of the product can be checked by TLC or by HPLC (see **Subheading 3.3**).

2—

Materials

1. Enzyme preparations: Some of the enzymes used in this procedure, e.g., myokinase from pig muscle (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40), and inorganic pyrophosphatase (EC 3.6.1.1), can be easily obtained from Sigma (Czech Republic, Prague) and/or from Boehringer (Mannheim, Germany), and it is advisable to do so since the enzymes are of a good quality and inexpensive. β -Galactosidase (bovine testes) (EC 3.2.1.23) can be obtained in very good quality from Boehringer, or it could be easily prepared following the published procedures (**16,19**). α 2,3-Sialyltransferase (pork liver) (EC 2.4.99.6) is available from Boehringer. However, its price is so high that for preparatory purposes, it is virtually inhibitory. To our knowledge, CMP-Neu5Ac synthase from calf brain (EC 2.7.7.43) is not commercially available.

2. All salts used for preparation of the buffers were of the highest purity (p.a. or better), NaCl for molecular biology. The quality of water is crucial—only bidistilled or Milli Q water can be used throughout all operations.

3. Protein concentration was determined by the Bio-Rad assay kit (Bio-Rad, Munich, Germany) using BSA as a standard. The procedure is described in the leaflet enclosed with the kit.

3—

Methods

3.1—

CMP-Neu5Ac Synthase from Calf Brain (EC 2.7.7.43)-Immobilized

3.1.1—

Materials and Solutions

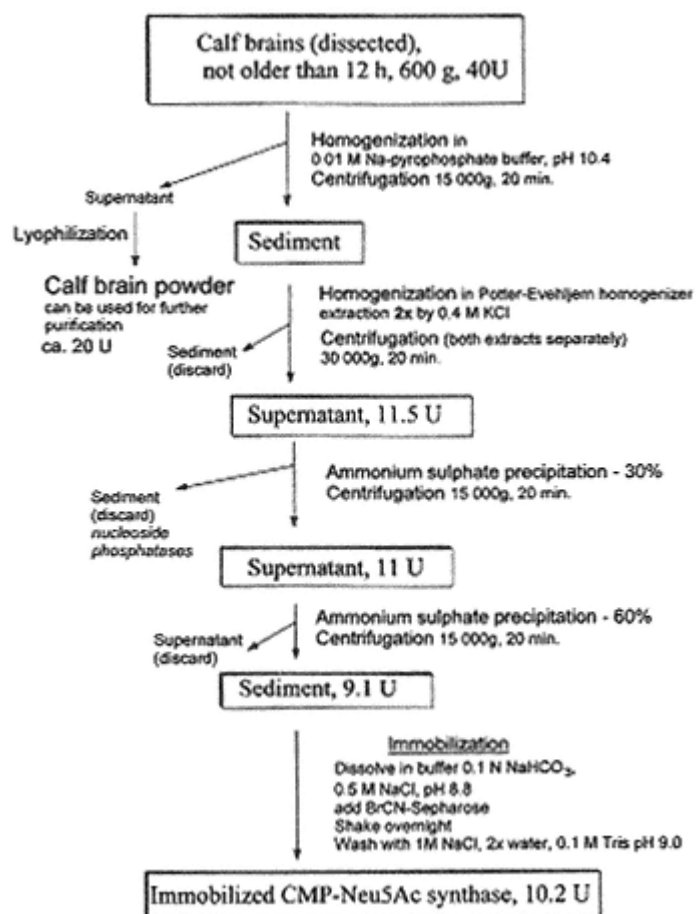
Extraction buffer A: 0.01 M Na-pyrophosphate, pH 10.4. Coupling buffer B: 0.1 M NaHCO₃, 0.5 M NaCl, pH 8.8. Storage buffer C: 0.1 M Tris-HCl, 3 mM 2-mercaptoethanol, pH 9.0.

3.1.2—

CMP-Neu5Ac Synthase Extraction and Purification1.

1. One calf brain (see Note ¹) (200 g, collected on ice on the slaughter, not older than 12 h) should be homogenized with 330 mL of extraction buffer A in the Waring blender (**Scheme 2**—modified according to [21]).

2. Centrifuge the homogenate for 20 min at ca 15,000g. Supernatant can be lyophilized and stored for further use as another source of the CMP-Neu5Ac synthase.



Scheme 2.

Purification of CMP-Neu5Ac synthase.

3. Extract the pellet twice with 200 mL 0.4 M KCl. The pellet should be homogenized in a Potter-Elvehjem homogenizer. The second extract usually has double the activity of the first one.
4. Centrifuge both extracts separately (20 min, 30,000g).
5. Precipitate each supernatant after centrifugation separately with $(\text{NH}_4)_2\text{SO}_4$ to 30% saturation (17.6 g/100 mL of the supernatant) (22). Add finely powdered $(\text{NH}_4)_2\text{SO}_4$ slowly under stirring at 0–4°C (ice bath) into the solution. After all the salt dissolves, the mixture should be stirred for another 15 min and then left to stand for 1 h in the ice bath.
6. Centrifuge the solution (20 min, 30,000g) and discard the pellet (containing mostly nucleosidases and phosphatases), bring the supernatant to 60% ammonium sulphate saturation (19.5 g $[\text{NH}_4]_2\text{SO}_4$ /100 mL), stir for 15 min, leave on ice

overnight, centrifuge (20 min, 30,000*g*), and discard the supernatant. The pellet consists of relatively pure synthetase (still containing some *O*-acetyltransferase activity).

3.1.3—

Immobilization of CMP-Neu5Ac Synthase

1. Preparation of CNBr-activated Sepharose 4B (Pharmacia) for the enzyme immobilization: required amount of the freeze-dried powder (1 g gives about 3.5 mL swollen gel) suspended in 1 mM HCl. The gel swells immediately, and should be slowly washed for 15 min with 1 mM HCl on a sintered glass filter. Use approx 200 mL/g dry powder.
2. Take up the enzyme precipitates, each in 55 mL coupling buffer B, add to the washed gel (each to 7 g gel-dry), and mix gently under nitrogen overnight.
3. The gel should be successively washed with 1 M NaCl, twice with distilled water, and finally with storage buffer C (blocks remaining active groups). The immobilized enzyme can be stored in the same buffer at 4°C. Immobilization often doubles the enzyme activity.

3.1.4—

Activity Determination of CMP-Neu5Ac Synthase

3.1.4.1—

Materials and Solutions

1. Tris-MgCl₂: mix 9 parts of Tris 1 M, pH 9.0, and 1 part 1 M MgCl₂.
2. Na-arsenite solution (highly toxic): 10% NaAsO₂ in 0.5 M Na₂SO₄ containing 0.05 M H₂SO₄.
3. Neu5Ac: 0.1 M 5-*N*-acetylneuraminic acid, pH adjust to 6.0 with KHCO₃ (store at -20°C).
4. CTP: 0.1 M CTP, adjust pH to 7.0 with KHCO₃ (store at -20°C).
5. 2-Thiobarbituric acid (TBA): 0.6% in water (heat to dissolve).
6. Na-metaperiodate: NaIO₄ 0.2 M in 9.0 M H₃PO₄.
7. NaBH₄: freshly prepared 2.7 M solution in ice-cold water.
8. Saturated solution of Na₂SO₄.
9. Acetone.
10. Cyclohexanone.

3.1.4.2—

Assay.

The assay (modified according to [23–25]) should be performed in the 10-mL centrifuge tubes to avoid transferring the resulting mixture prior to centrifugation.

1. Mix: Neu5Ac solution (25 µL), CTP solution (25 µL), enzyme (10–100 mU), Tris-MgCl₂ buffer (100 µL), and make up to 500 µL with water, incubate for 30 min at 37°C

2. Add 75 μL NaBH_4 solution, leave for 15 min, shake from time to time (this reagent stops the reaction and reduces unreacted Neu5Ac). The chemical reactions taking place in this assay are described in detail in the literature **(26)**.
3. Add 75 μL acetone, shake, and leave for 15 min (destroys unreacted NaBH_4).

4. Add 250 μL Na-metaperiodate solution, mix, and leave for 20 min
5. Add 1 mL Na-arsenite solution under mixing. Brown color appears, but after vigorous mixing, the solution becomes colorless.
6. Add 3 mL 2-thiobarbituric acid solution, and mix well by Vortex.
7. Place the tubes into the boiling water bath for 15 min. Pink color develops.
8. Transfer the tubes into the ice bath, add 0.5 mL saturated Na_2SO_4 , and leave for 10 min. Pink color fades, and a white precipitate appears.
9. Add 4 mL of cyclohexanone, vortex or shake intensively, and centrifuge shortly. The pink complex is extracted into the organic phase.
10. Absorbance of the organic phase should be measured against the blank (prepared with the boiled enzyme solution) at 549 nm. Absorption is linear up to 0.5.

3.1.4.3—

Calibration Curve Preparation

1. Into approximately seven tubes each add Neu5Ac solution (25 μL), CTP solution (25 μL), Tris-MgCl₂ buffer (100 μL), and make up to 500 μL with water.
2. Add NaBH₄ solution (75 μL), mix, and leave for 15 min.
3. Add 75 μL of acetone, mix, and leave for 15 min.
4. Into the tubes with the reduced Neu5Ac add 0–75 nmol Neu5Ac (0–75 μL of 1 M solution) and follow the above procedure, beginning with Na-metaperiodate addition.

One unit (1 U) of the enzyme incorporates 1 μmol of Neu5Ac into CMP-Neu5Ac within 1 h (this definition is common in the literature).

Activity calculation:

$$\text{Activity (U/mL)} = (c \cdot 60 / 10^3 \cdot t \cdot V) \quad (1)$$

c = amount of Neu5Ac (nmol) read from the calibration curve, t = reaction time (minutes), and V = volume of the sample (mL).

3.2—

α -2,3 Sialyltransferase (Pork Liver) (EC 2.4.99.6)

3.2.1—

Materials and Solutions

1. Glassware silanization: Fill a dry glass vessel to be silanized with the solution of 1% dichlorodimethylsilane (Aldrich) in dry (!) toluene, and leave for approx 10 min.
2. Empty the reagent, and rinse it with distilled water.
3. The glassware should be dried for 1 h at 80°C. The silanization solution can be used several times.

4. Waring blender (you may use kitchen blender with good-quality blades and plastic container), Amicon concentrator with PM-10 membrane.
5. CDP hexanolamine-Sepharose was obtained from M. Morr (GBF, Braunschweig, Germany). At present, this material is commercially available and it is marketed by Merck, Co. The gel is stored in 20% EtOH, and it contains 15–20 (mol CDP/mL. The gel is washed prior to use with water to remove EtOH and then with the respective buffer.
6. Buffer A: 25 mM Na-cacodylate (*see* **Note 2**), 20 mM MnCl₂, pH 6.0 (NaOH).

7. Buffer B: 25 mM Na-cacodylate, 20 mM MnCl_2 , 75 mM NaCl, 1.4% (w/v) Triton X-100, pH 6.0 (NaOH).
8. Buffer C: 10 mM Na-cacodylate, 25% (w/v) glycerol ($\rho = 1.26 \text{ kg/L}$), 0.1 M NaCl, 1% (w/v) Triton X-100, pH 6.0 (NaOH).
9. Buffer C: (variants with different NaCl concentrations) : 0.05 M NaCl, 0.5 M NaCl, 1.0 M NaCl, 1.5 M NaCl, 2.5 M NaCl.
10. Buffer E: 10 mM Na-cacodylate, 0.05 M NaCl, 0.5% (w/v) Triton X-100, pH 6.0 (NaOH).

3.2.2—

Cibacron Blue Agarose Preparation

1. About 100 g of agarose 6B (Sigma) (shipped in 20% EtOH) should be loaded into a column and washed well with distilled water to remove EtOH.
2. Suspend the gel in 350 mL H_2O in a 1000-mL conical flask.
3. Add a solution of Cibacron blue F3G-A (Fluka) 1 g/100 mL H_2O , and mix the slurry slowly on the rotary or reciprocal shaker (not the magnetic one-bead disruption!) for 5 min.
4. Add 50 mL of 20% (w/v) NaCl, and stir the mixture for another 30 min.
5. Add 2.5 mL of 5 N NaOH, and continue the mixing for 3 d.
6. Remove the liquid from the slurry by filtration through the paper on the Büchner funnel (weak suction) and wash the gel well with water, followed by 1000 mL of 1 M NaCl.
7. Then wash the gel with 1000 mL of 4 M urea (releases the unbound Cibacron blue) and then extensively with water until the eluate is colorless.
8. The affinity gel can be stored in water or in a respective buffer at 4°C.

In general, Cibacron blue agarose has broad selectivity for the enzymes utilizing phosphorylated substrates, nucleosides, and so forth (e.g., kinases).

3.2.3—

α -2,3 Sialyltransferase Extraction and Purification

The enzyme is purified from porcine liver following described methods (1,12,18,26–28) with some modifications.

It is essential that all operation are made in plastic vessels or in the siliconized glassware, including the columns (see **Subheading 3.2.1.**). Also use plastic tubes for fraction collection, because the enzyme adsorbs on the surface of normal glass. The ultrafiltration membranes and dialyzation tubes should be washed with water, and then incubated for 2 h in 1% bovine serum albumin and washed again with water to reduce the binding of the enzyme onto the membranes. All operations should be performed in a cooled room, and the samples should be kept on ice. Samples can be kept even at temperatures up to -5°C, since the buffers used contain glycerol or salt, but freezing (ice formation) must be excluded (**Table 1**).

1. Homogenize fresh pork liver (see **Note 3**) (280 g, kept on ice) in the Waring blender in buffer A (400 mL) (3×5 min strokes with a 10-min interval for cooling).

Table 1
Purification Protocol of Pork Liver α -2,3 Sialyltransferase

Purification step	Total volume, mL	Total activity, U	Specific activity, mU/amg prot.	Protein conc., mg/mL	Purif. factor	Total activity, (%)
Crude extract 1	370	5.0	1.55	8.7		
2	230	2.9	2.35	5.4		
Pooled	600	7.9	1.80	7.4	1	100
Cibacron blue agarose eluates						
1.0 M NaCl	340	0.94	2.69	1.026		
1.5 M NaCl	350	1.28	4.72	0.777		
Pooled	790	2.22	3.33	0.905	1.85	28
Dialysis, ultrafiltration	310	2.04	2.96	2.22	—	26
CDP-hexanolamine agarose eluates						
0.05 mM CTP	150					
		0.65	88.1	0.049		
0.1 mM CTP	150	0.60	67.5	0.059		
Pooled	300	1.25	77.8	0.54	43	16

2. Separate the membranes (see **Note 4**) by centrifugation (1 h, 5000g), and wash again with 250 mL buffer A using the same homogenization and centrifugation (**Scheme 3**).

3. Make the pellet up to 400 mL with buffer B, and add Triton X-100 solution (20%) to final concentration of 1.4%.

4. After homogenization, shake the mixture for 45 min and centrifuge (1 h, 5000g).

5. Repeat the extraction, and pool both extracts.

6. First Cibacron blue agarose 6B chromatography: load the pooled extracts on the column (plastic or siliconized) filled with Cibacron blue agarose 6B (4.5 × 12 cm) equilibrated with buffer C.

7. Wash the column with 500 mL buffer C, and then elute by a stepwise gradient of NaCl (0.5–2.5 M) in buffer C (350 mL steps with 0.5 M increments).

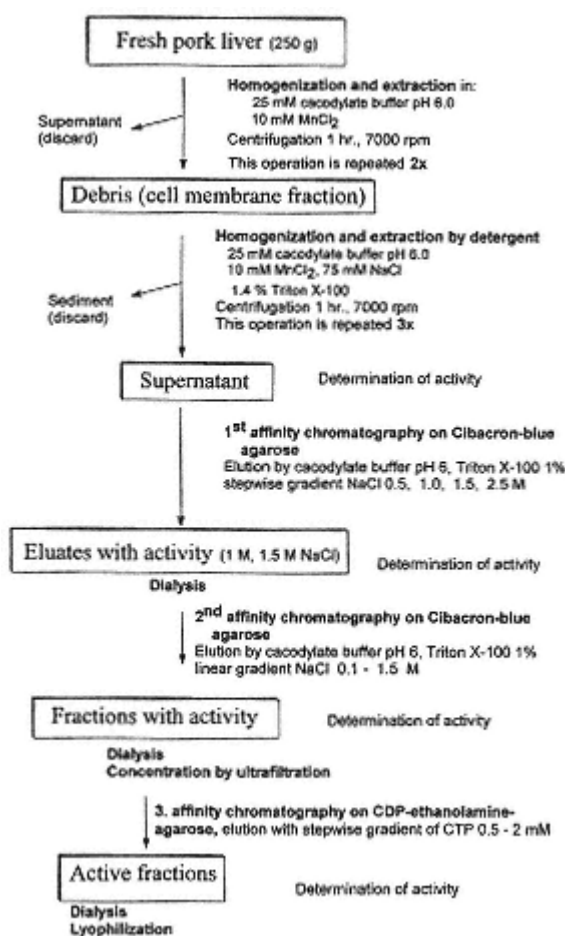
8. The fractions (5 mL) with 1 M and 1.5 M NaCl containing most of the α -(2,3 ST activity (assay) should be pooled and dialyzed two times against 4 L of buffer C without NaCl.

9. Second Cibacron blue agarose 6B chromatography (see **Note 5**): Load the active fractions from the first chromatography on the column and wash with 500 mL of buffer C.

10. Apply a linear gradient 0.5–2.0 M NaCl (gradient rate 0.1 M/50 mL) in buffer C.

11. Active fractions (around 1 M NaCl—but it is wise to assay the fraction) should be pooled and dialyzed two times against 4 L of buffer C without NaCl.

12. The volume of dialyzed fractions is reduced to half by ultrafiltration (Amicon concentrator with PM-10 membrane).



Scheme 3.
Purification of α -2,3 sialyltransferase.

13. Dialyze the fractions overnight against buffer D (4 L of buffer C containing 50 mM NaCl).
14. The combined fractions (see **Note 6**) should be divided into three parts (100 mL) and each of them applied to a column with 50 mL CDP-hexanolamine Sepharose 4B (see **Note 7**) equilibrated with buffer D.
15. Wash the column with 100 mL of buffer D followed by 100 mL of buffer E.
16. α -2,3 ST is eluted by a stepwise gradient (0.05–0.2 mM, 0.05-mM increments) of CTP in the buffer E (50-mL steps). Most of the activity can be found in the fractions 0.05 and 0.1 mM CTP (assay), which should be pooled and concentrated by ultrafiltration (membranes treated by BSA) to 50 mL.
17. The enzyme can be used either directly (storage at 0–4°C results in an approx 10% loss of the activity/wk) or dialyzed against buffer E containing 0.5% Triton

X-100. Resulting enzyme solution should be supplied with 0.15 mg BSA/mL and freeze-dried. This preparation is relatively stable up to 3 mo in a deep freezer. The dissolution should be done in a volume similar to the original one. Therefore, it is advisable to divide the enzyme into smaller vials prior to lyophilization (see **Table 1**).

3.2.4—

Sialyltransferase Activity Determination (Radioactive)

3.2.4.1—

Solutions and Materials

1. Aquasol-2 scintillation liquid (DuPont-New England Nuclear, Dreieich, Germany).
2. Scintillation flasks plastic (ca. 10-mL size).
3. Cytidine 5'-monophosphate sialic acid, (sialic-9-³H) (DuPont-New England Nuclear) (shipped as a solution in EtOH/water 7:3 in dry ice having an spec. act. of approx 17 Ci/mmol) (see **Note 8**).
4. Na-cacodylate buffer 0.25 M + 2% (w/v) Triton X-100, pH 7.5.
5. Bovine serum albumin (BSA) 5 mg/mL.
6. Na-phosphate buffer 5 mM, pH 6.9.
7. Galβ1–3GalNAc (Sigma) 25 mM.

3.2.4.2—

Preparation of Dowex 1 × 8-400/ PO_4^{3-}

1. The resin should be transformed into OH⁻ form by washing with 1 M NaOH (in the column) until strongly alkaline liquid elutes.
2. Wash extensively with water until neutral pH and then with 1 M H₃PO₄ (acidic eluate).
3. Finally wash extensively with water until neutral reaction and then extensively with the above Na-phosphate buffer (5 mM, pH 6.9). The resin can be stored in the same buffer in a refrigerator.
4. For the activity determination, fill Pasteur pipets with the resin.
5. Insert a small piece of cotton into the pipet, and pour the slurry of the resin into the pipet until a column of approx 3-cm height is formed. The pipets with the resin should be prepared shortly before the work. The resin must not be dry, but it can be drained off the buffer.

3.2.4.3—

Solution of Radioactive Labeled CMP-Neu5Ac.

1. Take 18 μL of solution of the radioactive CMP-Neu5Ac, 0.515 mg of nonradioactive CMP-Neu5Ac (Sigma), and dissolve in ca. 100 μL water.
2. Add 10 μL of the Na-cacodylate buffer, and make the solution up to 500 μL with water. The solution (radioactive!) must be stored in a deep freezer, preferably under -25°C. The solution contains 7 nmol of CMP-NeuAc in 5 μL, and this volume should have the activity of 40,000 counts/min (5% error is tolerable; check and adjust by dilution or activity addition).

Radioactivity is measured usually in counts/min. Theoretically, $1\text{ }\mu\text{Ci} = 2.22 \cdot 10^6\text{ counts/min}$, but β -radiation of ^3H has low energy, and therefore, the scintillation yield is only approx 40%. You should calculate the final activity

in your CMP-NeuAc solution according to the declared activity of the substance obtained.

3.2.4.4—

Activity Determination

1. Pipet into an Eppendorf tube: BSA solution (5 μ L), cacodylate buffer (5 μ L), Gal β 1–3GalNAc solution (5 μ L) (or water for the blank), enzyme (5 μ L), and CMP-Neu5Ac (5 μ L).
2. Mix and incubate for 15–30 min (depending to the enzyme activity) at 37°C.
3. Stop the reaction by addition of the phosphate buffer (1 mL, 5 mM, pH 6.9).
4. Pass the mixture immediately through the Pasteur pipet containing the Dowex 1 $\times$ 8-400/ PO_4^{3-} , and wash with another 1 mL of the same buffer.
5. Collect both eluates into the scintillation vial, and add the scintillation liquid Aquasol-2 (10 mL).
6. The activity should be counted in the β -counter set for tritium. The determination should be done in triplicate.

Activity calculation:

$$\text{Activity (U/mL)} = \frac{7 \cdot (\text{counts/min}_{\text{sample}} - \text{counts/min}_{\text{blank}})}{(\text{counts/min}_{\text{substrate}} \cdot V_{\text{sample}} \cdot t_{\text{incub}} \cdot 10^3)} \quad (2)$$

Where $\text{counts/min}_{\text{substrate}} = \text{counts/min}$ of 5 μ L solution of CMP-Neu5Ac (~ 7 nmol CMP-Neu5Ac) should be approx 40,000; V_{sample} (mL); t_{incub} (min).

One unit (1 U) of the enzyme incorporates 1 μ mol of the Neu5Ac into the product/1 min.

3.2.4.5—

Determination of α -2,3 Sialyltransferase Activity (Nonradioactive)

You may use this method (26) as an alternative to the radioactive one. It is quite easy to perform, quick, and no special instrumentation is needed. However, its sensitivity is somehow lower. There are some other nonradioactive methods for sialyltransferase determination, e.g., refs. (29–35). They, however, demand special chemicals and/or special instruments, e.g., HPLC.

3.2.4.5.1—

Reagents

1. Na-cacodylate buffer 0.25 M + 2% Triton X-100, pH 7.5.
2. BSA, 5 mg/mL.
3. Gal β (1 $\rightarrow$ 3)GalNAc, 25 mM.
4. CMP-Neu5Ac (Sigma) 1.05 mg/L mL Na-cacodylate buffer 25 mM, pH 7.5 (all three solutions above should be stored at -20°C).
5. Na-phosphate buffer, 5 mM, pH 6.9.
6. Na-metaperiodate, 0.2 M in 9.0 M H_3PO_4 .

7. Na-arsenite, 10% in 0.5 *M* Na₂SO₄ containing 0.05 *M* H₂SO₄.
8. TBA, 0.6% in water.
9. Saturated Na₂SO₄.
10. 1 *M* H₂SO₄.
11. Cyclohexanone.

3.2.4.5.2—

Typical Procedure (For α -2,3 ST)

1. Each assay mixture contains the following components: cacodylate buffer (25 μ L), BSA solution (25 μ L), Gal β (1 $\rightarrow$ 3)GalNAc solution (25 μ L), CMP-Neu5Ac solution (25 μ L), and enzyme (0.15–2 mU approx 25 μ L). Controls contain the heat-inactivated instead of the active enzyme or lack the acceptor substrate. The reaction should be started by the enzyme addition.
2. After incubation for 30 min at 37°C, the reaction is stopped by dilution with 1 mL of phosphate buffer, 5 mM, pH 6.9.
3. Pass the mixture immediately through the Pasteur pipet filled with Dowex 1 $\times$ 8, 200–400/ (PO₄)³⁻ (3 cm), and wash with 750 μ L of the same buffer.
4. The following procedure can be used for the identification of the active fractions containing sialyl-glycosides from the fractionation of the products by gel filtration—**Subheading 3.3.**
5. Add 1 M H₂SO₄ (170 μ L) to the combined elutes, and heat the mixture for 1 h at 80°C to hydrolyze the sialyl-conjugate.
6. The sialic acid released can be determined by modified TBA assay (36).
7. Add to the samples, cooled to room temperature, metaperiodate solution (350 μ L), and shake well.
8. After 20 min, add 1.5 mL of the arsenite solution, and shake the tubes until a yellow color disappears.
9. Add the TBA solution (3 mL), mix, and place the tubes in a boiling water bath for 15 min.
10. Remove the tubes and place them on ice for 5 min.
11. Then add 0.5 mL of saturated Na₂SO₄, and shake well. After cooling, the pink color fades and white precipitate appears.
12. After 5 min, add 4 mL of cyclohexanone, and vortex the tubes.
13. Centrifuge and collect the red organic layer, and measure its absorbance against the blank at 549 nm.

3.2.4.5.3—

Calibration Curve

1. Pipet 0–60 μ L of 1 M Neu5Ac neutralized by KHCO₃ (i.e., 0–60 nmol Neu5Ac) into the tubes.
2. Add 1.75 mL phosphate buffer (5 mM, pH 6.9), 170 μ L of 1 M H₂SO₄, and heat for 1 h at 80°C.
3. After cooling, the sialic acid can be assayed by the TBA method described above. The absorbances should be measured against the sample with no Neu5Ac. The calibration is linear up to 70 nmol Neu5Ac.

The overall sensitivity of the assay can be doubled when only 2 mL of cyclohexanone for extraction of the red complex are used. Both blank and calibration curve should be prepared with this modification. The sensitivity can be further increased by using a larger volume of the sample or by prolonging the incubation time.

Both methods—radioactive and nonradioactive—can be used for the other sialyltransferases determinations. You should use, however, a respective acceptor saccharide, e.g., lactose or *N*-acetyllactosamine.

3.3—

One-Pot Enzymatic Synthesis of SialylT-Epitope Procedure

1. Add to about 4 mL of water (see **Note 9**) (follow the sequence of the additions) Na-cacodylate buffer (1 mL; 0.25 M, pH 7.5, containing 2% of Triton X-100), 5-*N*-acetylneuraminic acid (70 μ mol; 0.7 mL of 0.1 M solution neutralized to pH 7.5 by NaHCO₃), CMP (50 mg, 100 μ mol), ATP-Na₂ salt (3 mg, 5 μ mol), CTP-Na₂ salt (0.3 mg, 0.5 μ mol), phosphoenolpyruvate K salt (300 mg, 200 μ mol), *N*-acetylgalactosamine (**1**) (150 mg, 0.7 mmol), *p*-nitrophenol- β -D-galactopyranoside (300 mg, 1 mmol), MnCl₂ (60 μ mol; 60 μ L of 1 M solution), MgCl₂ (0.12 mmol; 120 μ L of 1 M soln.), and KCl (0.2 mmol; 200 μ L of 1 M soln.). Dissolve and adjust pH to 7.5 (NaOH).
2. Add the following enzymes: myokinase from pig muscle (see **Note 10**) (EC 2.7.4.3, 600 U), pyruvate kinase (EC 2.7.1.40, 1200 U), inorganic phosphatase (pyrophosphatase) (EC 3.6.1.1, 5U), CMP-Neu5Ac synthase from calf brain immobilized on the CNBr Sepharose (EC 2.7.7.43, 1 U), β -galactosidase (bovine testes) (EC 3.2.1.23, 1.5 U) and α -2,3 sialyltransferase (pork liver) (EC 2.4.99, 0.1 U), and make the mixture up to 10 mL with water.
3. After 16 h of incubation at 37°C, add more *p*-NP-Gal (300 mg, 200 μ mol), β -galactosidase (0.7 U), and adjust pH to 7.5.
4. After the next 24 h, add Neu5Ac solution (40 μ mol), CMP-Neu5Ac synthase (0.2 U), and sialyltransferase (0.07 U). The addition of sialyltransferase (0.07 U) should be repeated after another 24 h, and the reaction can be terminated after total 82 h.
5. The reaction can be monitored by TLC using silica gel plates 60 GF₂₅₄ (Merck) with the solvent system *n*-propanol/water/ammonia 1 M (7:2:1 or 6:2:1). The spots can be visualized by UV light (only nucleosides), and all carbohydrate derivatives by charring with 5% H₂SO₄ in EtOH. For better separation, the plates should be developed twice (dry between the developments). Use galactose, *N*-acetylgalactosamine, Neu5Ac, and Gal β 1-3GalNAc as standards. The product (**3**) (not UV active) migrates slightly slower than Gal β 1-3GalNAc.
6. Alternatively, the product formation can be also monitored by HPLC (not tested) under following conditions: column RP18 (analytical), mobile phase: Na-phosphate buffer (15 mM, pH 5.2)/acetonitrile (19:1) or column Lichrosorb NH₂, mobile-phase Na-phosphate buffer (15 mM, pH 5.2)/acetonitrile (1:3–4), detection refractometric, or UV (220 nm).

3.3.1—

Product Separation

1. Dilute the reaction mixture twofold with water and add approx 7 mL of Dowex 1 $\times$ 2 (200–400 mesh) (PO₄)³⁻ equilibrated in 5 mM Na-P_i buffer, pH 6.9 (prepared as described in **Subheading 3.2.4.2.**).

2. Collect the supernatant after 20 min, and wash the ion-exchange resin with 15 mL of the equilibration buffer. This treatment removes most of the free Neu5Ac, CMP-Neu5Ac, and *p*-nitrophenol.
3. Concentrate the pooled eluates by freeze drying to approx 2 mL and apply to the column (4×100 cm) of BioGel P2 (200–400 mesh) equilibrated with water (see **Note 11**).
4. Elute the column with water. Refractometric detection shows tentative position of the active fractions. However, respective fractions must be assayed by the TBA after hydrolysis with H₂SO₄.
5. Take 100 to 200-μL samples and follow the procedure as described in **Subheading 3.2.4.5.2. (25)**. This helps to localize exactly the fractions containing the sialylated product. TLC can be used to check the purity of the fractions (sample concentration by freeze-drying is often necessary).
6. Then the active fraction should be pooled and lyophilized to give 27 mg of product **3**. The isolated yield of the reaction was 26 mg of **3** (36%), and analytical yield determined at the end of the reaction was 45% (related to Neu5Ac) (**5**). The identity of the product can be confirmed by ¹H NMR and/or by mass spectrometry.

4—

Notes

1. You should consider all bovine brain material as potentially infectious in the view of the recent occurrence of Creutzfeld-Jakobs disease.
2. Cacodylic acid (dimethylarsinic acid) and its salts are very toxic, and they are potential human carcinogens. Gloves must be used throughout all operations.
3. It is advisable to go personally to the local slaughterhouse to ensure that good quality pork liver is collected on ice immediately upon a slaughter. Extraction and first Cibacron blue Sepharose chromatography can be then done within the first day. This ensures very good enzyme yield and a high specific activity.
4. Sialyltransferase is a membrane-bound enzyme, and it is released by detergent—Triton X-100 addition. The presence of the detergent is also essential for its activity in vitro.
5. Second Cibacron blue agarose 6B chromatography can be skipped. However, you may (in the case when CDP-hexanolamine Sepharose is not available or if you have only a limited amount of it) even use the enzyme preparation from the second chromatography directly for carbohydrate sialylation. After the second chromatography also, a lower amount of the affinity carrier can be used.
6. It is essential that the concentration of NaCl in the sample to be loaded on the column is not higher than 60 mM, since NaCl in conc. of about 0.1–0.15 M blocks completely the enzyme binding to the affinity carrier.
7. The capacity of the affinity carrier is about 1 U of the enzyme/50 mL of gel.

8. You will work with the low-energy β -radiation. No special measures against irradiation are necessary, since it is mostly absorbed by the glass of the vessels. However, surface contamination and especially internal contamination are very dangerous. You must work in a fume hood with good ventilation (check with a strip of paper) on the plastic tray. Use gloves, and separate all contaminated

material according to the local regulations. The contamination of materials and instruments cannot be measured with a Geiger counter but you must take the surface samples with the wet swabs and measure their scintillation.

9. The water should be sterile. Semisterile work is advisable to avoid bacterial contamination during the work. Some authors recommend working also under argon (or N₂) atmosphere.

10. It may happen that the reaction does not proceed because of problems with the CTP regeneration. Then try to add a small amount of CTP or you may try an alternative system, e.g., nucleoside monophosphate kinase from yeast (37).

11. Careful packing of the column is crucial for good fractionation—follow the instructions provided by the producer. BioGel P2 shows weak ionic interactions that cause migration of the charged species not exactly according to their molecular masses, e.g., sialyl glycosides and Neu5Ac migrate faster than neutral trisaccharides, nitrophenol remains detained on the column much longer (diffuse yellow band) than expected from its molecular mass, and so forth.

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15—

Hydrolysis of Hemicelluloses Using Combinations of Xylanases and Feruloyl Esterases

Craig B. Faulds, Paul A. Kroon, Begoña Bartolomé, and Gary Williamson

1—

Introduction

1.1—

Hemicelluloses in the Plant Cell Wall

Hemicelluloses are heteropolysaccharides that occur in many plant cell walls. Usually hemicelluloses consist of a xylan backbone highly substituted with sugar side chains and with acetyl, feruloyl, coumaroyl, and other groups; the polymer is linked to protein, cellulose, and other cell wall components. The hemicellulose component of the cell wall helps prevent infection, provides strength, and protects against other external agents. Plant pathogens hydrolyze the plant cell wall, including the hemicellulose component, prior to invasion, and dead plant cell walls are degraded by Saprophytic fungi and other microbes to utilize the components as energy. This digestive process also occurs in ruminants and in the colon of humans, and is catalyzed by gut microflora.

1.2—

Phenolic Content

Plant cell walls contain covalently bound phenolics. The most abundant is ferulic acid, which is ester-linked to arabinosyl residues as part of hemicellulose. Some materials such as wheat **(1)** or maize bran **(2)** contain a high content of ferulic acid (~0.8 and ~3% w/w, respectively). Another phenolic-rich component, lignin, may also be attached to hemicellulose via other hydroxycinnamic acids such as *p*-coumaric acid. These phenolics affect the properties of the plant cell wall.

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1.3—

Why Hydrolyze Hemicellulose?

On an industrial scale, hydrolysis of hemicelluloses by xylanases and ferulic acid esterases constitutes an environmentally friendly way to obtain high added-value compounds such as ferulic acid and monosaccharides from waste or low-value materials (e.g., cereal brans as waste products from milling and barley spent grains from the brewing process). Treatment of certain animal feedstuffs with xylanases and esterases may also improve their nutritional value. Analytically, enzyme hydrolysis is helpful in elucidation of plant cell wall structural features. Since xylanases and esterases show different substrate specificities, these enzymes can be used as analytical tools for determining different structural features (e.g., frequency, position) of the substitutions in the hemicellulose. Also, the hemicellulose component can be specifically removed using xylanases and esterases, and the remaining plant cell wall analyzed further. Enzymic hydrolysis allows preparation of lower molecular weight oligosaccharides to give information on the cell wall linkages or used for other applications such as gelation.

1.4—

Alkali/Acid and Enzymic Hydrolysis

Plant cell walls may be exposed to extremes of pH to break certain linkages within the cell wall. Alkali treatment breaks the ester bond linking phenolic acids to the cell wall. Acid treatment breaks glycosidic bonds and solubilizes sugars while leaving ester bonds generally intact. These chemical treatments, however, require high concentrations of acid or base, and such treatments lead to modifications of other components and additional (and often unwanted) chemical changes in the plant cell wall. A gentler and more specific method is to use enzymatic hydrolysis.

For complete enzymatic hydrolysis of hemicelluloses a mixture of enzymes is required, such as glycanases, β -glycosidases, and esterases. Xylanases break down the xylan backbone to short xylo-oligosaccharides, and then xylosidases break these intermediates to xylose. Substitutions on the backbone reduce or block xylanase action, and hydrolysis can only proceed if these side chains are removed. Arabinose is the most common substitution in many xylans, and gives rise to the name arabinoxylan. Arabinose groups and arabinose chains are linked on C-2 and C-3 and can be substituted with other groups. Acetyl groups are located at the C-2, C-3, or on both C-2/3 positions of xylose (3). Ferulic acid is covalently linked through an ester bond to the C-5 position of arabinose residues in cereals (4,5). Enzymes in the growing plant cell wall convert some of the ferulic acid to diphenolic crosslinks, which play a structural role in maintaining the integrity of the cell wall and forming a defensive barrier against microbial attack.

1.5—

Interactions Between Enzymes

Xylanases often show a highly synergistic interaction with other main chain cleaving enzymes such as β -xylosidases, and with side chain cleaving enzymes such as α -L-arabinofuranosidases, acetyl xylan esterases (AXE), and ferulic acid esterases (FAE). A synergistic interaction occurs when the amount of product formed by the action of two or more enzymes in the same reaction exceeds the sum of the products formed by the action of the individual enzymes. Examples of synergy include the breakdown of heteroxylans by the action of glycanases and esterases, e.g., glucuronidase/AXE on beechwood glucuronoxylan (**6**), xylanase/FAE on wheat (**7,8**), xylanase/AXE on birchwood acetyl xylan (**9**), and xylanase/acetyl glucomannan esterase on galactoglucomannan (**10**). However, steric hinderance may affect the reactivity of xylanases and esterases where it has been shown that the esterase will cleave the ester bond, but other factors restrict the access of the enzymes to the substrates (**11**). Another factor is the position of the ester link on the primary sugar, where certain linkages may not be hydrolyzed by a specific esterase (**12**).

1.6—

Breakdown of Hemicelluloses.

To break down Hemicelluloses using xylanases and esterases, there are several steps involved in performing an experiment:

1. Enzymatic hydrolysis (see **Subheadings 2.1.** and **3.1.**).

- a. Preparation of hemicellulose-containing substrate.
- b. Isolation of enzymes and determination of purity.
- c. Hydrolysis and termination of the reaction.

2. Phenolics analysis (see **Subheadings 2.2.** and **3.2.**).

- a. Extraction with ethyl acetate.
- b. Separation by reverse-phase high-performance liquid chromatography (HPLC).
- c. Analysis of data.

3. Sugars analysis (see **Subheadings 2.3.** and **3.3.**).

- a. Sample preparation.
- b. Separation by high-performance anion-exchange chromatography.
- c. Analysis of data.

2—

Materials

2.1—

Enzymatic Hydrolysis

1. Cell wall preparations. It is beyond the scope of this chapter to give a detailed account of the various methods for preparing cell walls and isolating cell wall components; therefore, the reader is referred to Selvendran and Ryden (**1**) (*see also* **Note** [1](#)). Examples of cell walls containing feruloyl esterified hemicellulose are cereal brans and straws (e.g., wheat and maize), bamboo shoots, barley spent

grain, and Chinese water chestnut. Cell walls can be pretreated or used intact depending on the experimental design and purpose. Examples of pretreatments include destarching, milling to obtain fine powders, or sieving to obtain a range of particle sizes. Cell-wall preparations should be dried in an oven for 24 h (60°C) before use so that reproducible water contents are obtained.

2. Purified xylanase(s). Purified enzymes can be purchased commercially from a specialized company such as Megazyme (Co. Wicklow, Ireland) (e.g., *Trichoderma viride* xylanase is >99% pure, and beware of companies selling polysaccharide-degrading enzymes which are actually crude culture supernatants from microbial cultures), or purified from microorganisms or plants (see **Note 2**). Driselase (Sigma-Aldrich, Poole, Dorset, UK), a *Basidiomycetes* enzyme preparation, is a useful tool for degradation of cell walls since it contains several *exo*- and *endo*-hydrolases (including β -xylanase, cellulase, pectinase, and β -mannase), but is completely free of FAE activity. Driselase can be used to release most of the feruloyl groups from plant cell walls as small, soluble feruloylated oligosaccharides, which are good substrates for FAEs. Driselase should be purified from contaminating phenolic compounds before using it by a simple published method (**14**).

3. Purified feruloyl esterase. This enzyme is not yet commercially available in a pure form and must be isolated from a microbial source or from appropriate commercial enzyme mixtures (see **Note 2**). Feruloyl esterases have been identified from both bacterial (**15,16**) and fungal sources (**17,18**), and examples of how to purify these enzyme can be found in the appropriate references. Feruloyl esterases have also been purified from cellulase and pectinase preparations (**19**).

4. Hydrolysis buffer. The choice of buffer salt, strength, and pH should be based on the pH optimum and buffer tolerance of the xylanase(s) and FAE(s) used. The pH optimum of the esterase and xylanase may not be similar, so a compromise may be required. We have used 100 mM (3-[N-Morpholino] propane sulfonic acid) (MOPS) buffer at pH 6.0 or 50 mM citric acid- Na_2HPO_4 (McIlvaines buffer; pH range 2.6–7.6). Water may also be used. Hydrolysis buffers should always contain 0.04% (w/v) sodium azide (antimicrobial agent; see **Note 3**).

5. Aqueous sodium hydroxide (1 M) and glacial acetic acid.

6. A thermostatically controlled rotating incubator (4 rpm) or rotating vertical mixer in an incubator. This should give reproducible agitation and allow the enzyme to interact better with the (insoluble) substrate without substantial physical breakdown of the substrate (see **Note 4**).

2.2—

Analysis of Phenolics

1. Internal standard solution for HPLC. Dissolve 14.82 mg cinnamic acid in 100 mL methanol (=1 mM stock); solution is stable for 6 mo if kept in the dark at -20°C. On the day of use, prepare a 10 nmol/mL solution by slowly adding 1 mL of the 1 mM cinnamic acid stock to 99 mL water while stirring (see **Note 5**).

2. Ethyl acetate, sovril tubes, a benchtop centrifuge which handles sovril tubes, and pasteur pipets for extracting phenolics.

3. A sample concentrator or equivalent (e.g., stream of nitrogen gas through pasteur pipet) for evaporation of ethyl acetate.
4. A solution of methanol-water (50:50, v/v) and 0.2- μm sample filters (e.g., Minisart RC 4, Sartorius, Epsom, Surrey, UK). The MeOH-water mixture should be degassed before use (see **Note 6**).
5. An HPLC system capable of running gradients coupled to either a diode-array detector (e.g., Gilson 160 ultraviolet (UV)/Vis diode-array detector) or a variable wavelength UV detector capable of measurements at 280 nm and 310 nm (see **Note 7**).
6. A C-18 reverse-phase HPLC column for separation of phenolics (we use an Inertpak ODS-2, 25 cm $\times$ 5 mm id, 5 μm ; Capital HPLC Ltd, Broxburn, West Lothian, UK).
7. Buffers for separation of phenolics by HPLC. Buffer A, 100 mL acetonitrile plus 72 μL trifluoroacetic acid made up to 1 L with water; buffer B, 400 mL acetonitrile plus 400 mL methanol plus 7 (77 μL) trifluoroacetic acid made up to 1 L with water. Acetonitrile (far-UV quality), methanol (HPLC quality), and water (MilliQ quality, 18.2 $\text{M}\Omega\text{ cm}^{-1}$, or equivalent) should be filtered (0.2 μm) before mixing. Buffers should be degassed thoroughly before use (see **Note 6**).
8. Appropriate phenolic acid standards. Simple phenolics (e.g., ferulic, vanillic, *p*-coumaric, sinapic and caffeic acids, vanillin, and *p*-hydroxybenzaldehyde) can be purchased from chemical suppliers such as Sigma-Aldrich. Less common and less abundant phenolics such as dehydrodimers of ferulic acid should be synthesized (see **ref. 20**) or purified from appropriate sources (e.g., wheat/maize brans, sugar-beet pulp) using preparative HPLC. Standard stock solutions (1 mM) should be prepared in methanol and can be stored at -20°C for 6 mo (see **Note 5**).

2.3—

Sugars Analysis

1. An HPLC system capable of running gradients coupled to a Dionex triple-pulsed amperometric detector (PAD) for high-performance anion-exchange chromatography (HPAEC). The reader is encouraged to study the Dionex Technical Note (analysis of carbohydrates by anion-exchange chromatography with pulsed amperometric detection, 1989; Dionex, Camberley, Surrey, UK), the review on HPAEC for carbohydrate analysis by Lee (**21**), and a description of the method used here (**22**) before proceeding.
2. A column for HPAEC (e.g., CarboPac PA-100 pellicular anion-exchange resin column, 25 cm $\times$ 4 mm).
3. Buffers for separation of carbohydrates by HPAEC (see **Note 8**). Buffer A, 145 mM aqueous NaOH (11.6 mL 50% sodium hydroxide solution [BDH, Poole, Dorset, UK] made up to 1 L with filtered [0.2 μm] MilliQ or equivalent quality water); buffer B, 500 mM sodium acetate in 145 mM aqueous NaOH (dissolve 41.02 g sodium acetate in 994 mL MilliQ or equivalent quality water, filter [0.2 μm] and add 11.6 mL 50% NaOH).
4. Appropriate monomeric sugars and oligosaccharide standards. Purified xylo-, galacto-, and arabino-oligosaccharides (up to octamers) can be purchased from

Megazyme. Simple sugars are available from most major chemical companies. Prepare standards on the day of use by dissolving the oligosaccharides (dried to constant weight) in 145 mM sodium hydroxide to give a 10-mM (10 μ mol/mL) stock solution. Dilute the stock solution with 145 mM sodium hydroxide and inject 20, 40, 60, 80, and 100 nmol to calibrate the column.

3—

Methods

3.1—

Enzymatic Hydrolysis

1. Weigh out 10.0 ± 0.1 mg portions of cell wall material into 1.5 mL Eppendorf tubes. Triplicate samples should be prepared as a minimum (see **Note 9**) for each treatment including controls (enzyme solution boiled for 5 min and cooled prior to addition of cell wall material).
2. Add water or buffer and enzyme(s) to a final vol of 1.0 mL.
3. Incubate the mixture in a thermostatically controlled rotating incubator (see **Note 4**). The tubes should be protected from light, which can cause isomerization and polymerization reactions with phenolics.
4. Terminate reactions by boiling the samples for 5 min. Allow to cool and centrifuge (13,000g for 5 min) to sediment the insoluble material.
5. Split the soluble component of the samples into 2 parts by removing duplicate portions of 0.4 mL to clean sovril tubes. One part is analyzed for phenolics (see **Subheading 3.2.**) and the other for sugars (see **Subheading 3.3.**).
6. Determine the amount of alkali-extractable phenolics (=esterified) by adding NaOH (1 mL of 1 M) to 10.0 mg triplicate portions of cell wall material and incubating at room temperature in the dark. After 24 h incubation, centrifuge (13,000g for 5 min) to sediment insoluble material, and remove portions (0.4 mL) to clean sovril tubes for phenolics analysis (see **Subheading 3.2.**).

3.2—

Analysis of Phenolics

1. Add 10 nmol of a suitable phenolic acid to the sample (0.4 mL) as an internal standard. Cinnamic acid (1 mL of a 10 nmol/mL stock solution in water) is recommended since this phenolic is well separated from the common cell wall phenolics under the HPLC conditions described here.
2. Add 100 μ L of glacial acetic acid to the sample and vortex mix. The pH of the solution should be about 3.0 and should be tested with pH paper. The low pH ensures that phenolics are in the acid form and will be efficiently extracted into the organic phase (see **Subheading 3.2.**).
3. Extract the aqueous phase with ethyl acetate (see **Note 10**). Add 3 mL ethyl acetate to the aqueous solution, mix thoroughly (vortex mixer for 20 s) and allow the aqueous and organic layers to separate. A short centrifugation step (2000g for 5 min) will accelerate layer separation. Remove the upper organic layer to a fresh sovril tube and repeat the extraction of the aqueous phase twice more, each time with 3 mL ethyl acetate.
4. Take the pooled organic fractions to dryness using a stream of inert gas (e.g., nitrogen) while the sovril tubes are warmed (40°C) in a dry-block or in a beaker of water.

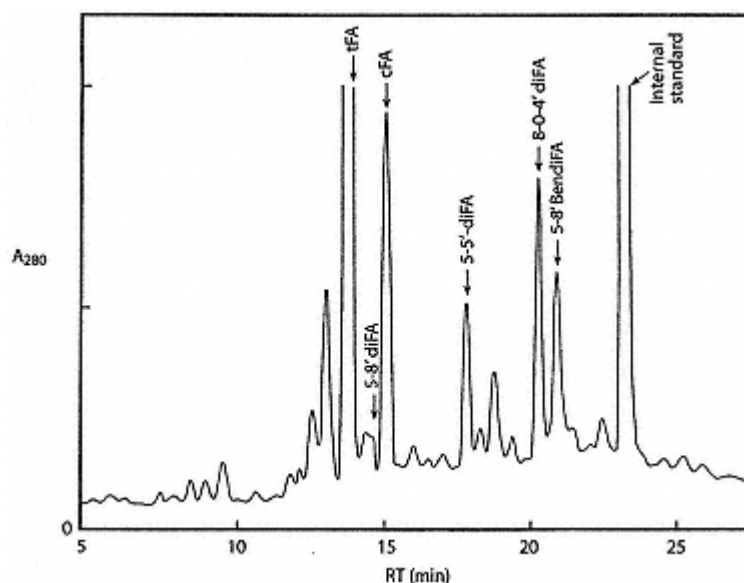


Fig. 1.

HPLC chromatogram (at 280 nm) of the supernatants of destarched wheat bran (0.01 g) after sodium hydroxide (1 M) hydrolysis at 20°C for 16 h under nitrogen gas. *trans*-Cinnamic acid was used as the internal standard. Peaks were identified by comparing the retention times and spectra to those obtained for pure standards. Abbreviations: tFA, *trans*-ferulic acid; 5-8'diFA, (E,E)-4,4'-dihydroxy 3,5-dimethoxy- β -3-bicinnamic acid; cFA, *cis*-ferulic acid; 5-5'diFA, (E,E)-4,4'-dihydroxy-5-5'-dimethoxy-3-3'-bicinnamic acid; 8-O-4'diFA, (Z)- β -(4-[(E)-2-carboxyvinyl]-methoxyphenoxy)-4-hydroxy-3-methoxycinnamic acid; 5-8'bendiFA, *trans*-5-[(E)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid; RT, retention time.

5. Redissolve the dried samples in a mixture of methanol-water (50:50, v/v; 1 mL) and filter (0.2 μ m cellulose membrane, e.g., Sartorius Minisart RC 4) the sample into HPLC vials.

6. Separate phenolics (10 to 100 μ L portions) by HPLC using a C-18 reverse phase analytical column. Elute the column at 1 mL/min with the following gradient system that increases the concentration of methanol and acetonitrile in 1 mM aqueous trifluoroacetic acid (TFA) using solvent A (10% aqueous acetonitrile in 1 mM TFA) and solvent B (40% methanol/40% acetonitrile in 1 mM TFA): initially 90% A, 10% B; linear gradient over 25 min to 25% A, 75% B; linear gradient over 5 min to 10% A, 90% B; held isocratically at 10% A, 90% B for 5 min. Detect phenolics using a diode-array detector or a UV detector at 280 nm (see **Note 7**).

7. Integrate the peak areas at 280 nm and use the retention time and accumulated spectral data (if using diode-array detector) to confirm the identity of each peak (23) (see **Fig. 1** and **Note 11**).

8. Quantify peaks by reference to calibrations made by eluting known amounts of pure compounds (range 1–10 nmol) including internal standard on the HPLC. Dilute phenolic acid stock solutions with MilliQ water (see **Note 5**) and obtain a standard curve with at least 5 points. Plot peak area (y-axis) against nmol (x-axis) and calculate the slope (peak area/nmol; Slope_{std}). We have found phenolic acid standard curves are linear up to 50 nmol/peak, except for caffeic acid, which gives an exponential curve.

9. Calculate the amount of phenolic acid released enzymatically from the cell wall sample as follows:

- a. Calculate the peak area for phenolic release due only to enzyme activity (Area_E) by subtracting the mean value for the appropriate blank sample (Area_B) from the mean value for the enzyme-treated sample (Area_S):

$$\text{Area}_E = \text{Area}_S - \text{Area}_B \quad (1)$$

- b. Calculate the amount of enzymic phenolic acid release from the starting material (10 mg) using the following equation:

$$\text{Phenolic (nmol)} = (\text{Area}_E / \text{Slope}_{\text{std}}) \times (1000/V_i) \times 2.5 \quad (2)$$

where V_i is the volume (in μL) of sample injected onto the HPLC, and 2.5 is a factor to correct for the removal of 400 μL for phenolics analysis from a total vol of 1 mL.

There are various reasons why there may be a lack of solubilized phenolics following enzymatic hydrolysis (see **Note 12**).

- c. The amount of alkali-extractable phenolic acid (see **Subheading 3.1.6.**) can also be calculated using **Eqs. 1 and 2.**

3.3—

Sugars Analysis

1. Prepare samples (0.4 mL) for HPAEC by adding 70 μL 1 M NaOH solution.

2. Analyze low-molecular-mass carbohydrates (see **Note 13**) using HPAEC coupled to a PAD. Separate carbohydrates (10–100 μL portions) using a pellicular anionexchange resin column eluted isocratically at 1 mL/min with the following gradient system, which increases the concentration of sodium acetate in 145 mM aqueous sodium hydroxide using solvent A (145 mM aqueous NaOH) and solvent B (500 mM aqueous sodium acetate in 145 mM NaOH): initially 90% A, 10% B; held isocratically at 90% A, 10% B for 10 min; linear gradient over 30 min to 0% A, 100% B; held isocratically at 0% A, 100% B for 10 min; linear gradient over 10 min to 90% A, 10% B; held isocratically at 90% A, 10% B for 10 min.

3. Integrate peak areas and use the retention time to identify each peak.

4. Quantify peaks by reference to calibrations made using known amounts of pure compounds (0–100 nmol; see **Note 14**). Dilute oligosaccharide stock solutions with MilliQ water and obtain a standard curve with at least five points. Plot peak area (y-axis) against amount (μg ; x-axis) and calculate the slope (peak area/nmol; Slope_{std}). We have found oligosaccharide standard curves are linear up to 100 nmol/peak.

5. Calculate the amount of each sugar/oligosaccharide released enzymically from the cell wall sample as follows:

- a. Calculate the peak area for sugar release due only to enzyme activity (Area_E) by subtracting the mean value for the appropriate blank sample (Area_B) from the mean value for the enzyme-treated sample (Area_S):

$$\text{Area}_E = \text{Area}_S - \text{Area}_B \quad (3)$$

- b. Calculate the amount of enzymatic sugar release from the starting material (10 mg) using the following equation:

$$\text{Sugar (nmol)} = (\text{Area}_E / \text{Slope}_{\text{Std.}}) \times (470 / V_i) \times 2.5 \quad (4)$$

where V_i is the volume (in μL) of sample injected onto the HPAEC, and 2.5 is a factor to correct for the removal of 400 μL for phenolics analysis from a total volume of 1 mL.

4—

Notes.

1. Ester bonds are alkali-labile, so extraction of cell walls by alkaline treatments ($\text{pH} > 10$) must be avoided since this will result in the loss of nearly all esterified phenolic groups.
2. Xylanases exhibit highly synergistic relationships with debranching activities (e.g., α -L-arabinofuranosidase, acetyl xylan esterase, and feruloyl esterase) in the hydrolysis of hemicelluloses. Isolated xylanases and feruloyl esterases should be assayed carefully for these activities before they are accepted as “pure.” There are a range of xylanases that exhibit different hydrolytic patterns on particular hemicellulose substrates, and hence produce a different range of products. The preference of feruloyl esterases depends mostly on the size of the substrate (small feruloylated oligosaccharides are best) and the linkage between the phenolic and primary sugar (12,25).
3. Always ensure that an antimicrobial agent is present in the assay buffer since microbes can utilize sugars and phenolic acids as carbon sources. Sodium azide (0.02–0.04 %) is recommended, but should be treated with care due to its toxicity.
4. Mixing is required to allow the enzymes access to the surface of the substrate by maintaining the insoluble cell wall polysaccharides in a suspension. However, if the mixing process is too vigorous, there may be physical damage to the cell walls which may release some components as soluble poly- or oligosaccharides. This will give an increase in soluble sugars and will appear to be because of enzyme activity. It is important to avoid this problem and to perform appropriate controls (substrate plus buffer with mixing, no enzymes) to assess the amount of nonenzymatic solubilization.
5. Phenolic acids are readily soluble in methanol but possess low solubility in aqueous solution at low pH. The phenolic acid must be fully dissolved before use, and should be heated (90°C , 10 min) if crystals are visible.
6. Mixtures of methanol and water should be degassed before they are used for HPLC. Continuous helium-purging is the best method, but other methods such as sonication and vacuum degassing will suffice.

7. The choice of detector depends on the absorption characteristics of the phenolic acids of interest. A diode-array detector is ideal because it gives a spectrum at each time unit and can be used for peak identification. Fixed and variable wavelength UV/Vis detectors can be used for the more abundant phenolic acids (ferulic/*p*-coumaric) with careful choice of wavelength; we recommend 280 nm since most phenolics absorb at this wavelength. However, the response factors and detection limits for each phenolic acid are strongly dependent on the wavelength (λ) used; for increased sensitivity, a wavelength at or near the λ_{max} of the phenolic of interest should be used. For the detection and identification of less abundant phenolics such as ferulic acid dehydromers, a diode-array detector and pure standards are essential; a variable wavelength UV detector is not suitable for this purpose.

8. To prevent (1) dust entering the buffers (causing fouling of HPLC filters and columns) and (2) sodium carbonate precipitating in the sodium hydroxide solution, do not agitate the stock and remove portions only from the central, top part of the bottle. Purchase fresh sodium hydroxide every 6 mo. Sodium hydroxide pellets are not suitable since they are covered in a film of sodium carbonate which is a strong eluent or “pusher.”

9. Plant cell wall preparations can be rather heterogeneous, especially when the fragment size is large. Each sample should, therefore, be analyzed at least in triplicate. Preferably, more than one cell wall sample is prepared and each sample analyzed in triplicate so the variation can be estimated.

10. Only free phenolic acids are separated efficiently into the organic layer in this process, and not esterified phenolic acids (e.g., feruloylated oligosaccharides such as FAXX; **14**) which may also be present in the reaction mixture. In plant cell walls, ferulic acid is present almost exclusively as the *trans* isomer. However, isomerization occurs during the extraction process to yield significant quantities of the *cis* isomer. The total amount of *trans*-ferulic acid in the cell wall can be calculated by addition of the values for the *trans* and *cis* isomers after extraction. Samples analyzed without extraction into ethyl acetate should contain virtually none of the *cis* isomer, but this will shorten the life of HPLC columns.

11. Detection limits should be determined for both HPLC analysis of phenolics and HPAEC analysis of carbohydrates. It is usual only to integrate peaks that give a peak height at least three times that observed for baseline noise; this should be used to calculate detection limits.

12. HPAEC separation is performed in alkaline conditions (145 mM NaOH), which will rapidly cleave ester bonds between phenolics and sugars. Esterified phenolic oligosaccharides will be degraded so that only the carbohydrate moiety is detected by PAD during HPAEC separation.

13. Some FAEs require the presence of a xylanase to release phenolic acids from plant cell walls (**15,24**), while others are able to release small quantities of phenolics when acting alone (**7,25**). The position of the ester-link between ferulic acid and the primary sugar can also influence hydrolysis; in monocots the ester

bond is through the C-5 of arabinose, but in dicots (sugarbeet, spinach) the bond is to either the C-2 of arabinose or the C-6 of galactose. Some feruloyl esterases are specific for the location of the ester bond (**12,25**). Some cell wall substrates are very complex and difficult to hydrolyze, even with combinations of xylanase and FAE, or with Driselase and FAE (**11**). This is due to steric hindrance, i.e., enzyme access to cleavage sites is restricted by the number and complexity of substitutions on the xylan backbone.

14. There are various methods commonly in use for measuring sugars. These include colorimetric assays for reducing groups (e.g., dinitrosalicylic acid reagent; **ref. 26**) and for total sugars (e.g., phenol sulfuric acid reagent; **ref. 27**), and gas chromatographic methods (**28**).

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16—

Enzymatic Depolymerization of Chitins and Chitosans

Riccardo A. A. Muzzarelli, Vesna Stanic, and Viviana Ramos

1—

Introduction

The enzymatic depolymerization of chitin has been explored in consideration of the disadvantages of the chemical hydrolysis, namely partial deacetylation of the resulting oligomers, difficulty of controlling depolymerization, low yield, cumbersome isolation of oligomer mixture, and heterogeneous conditions. Preparation of chito-oligomers by enzymatic depolymerization does not produce deacetylation. It would seem that the most obvious approach to the production of chito-oligomers is the use of chitinases, but the comparative evaluation of the data obtained with other enzymes points to a different conclusion. In this chapter, the following depolymerization methods are presented and discussed: (1) chitinase on chitin, (2) chitosanase on chitin, (3) chitosanase on chitosan, (4) hemicellulase on chitosan, (5) lysozyme on chitosan, (6) papain on chitosan, and (7) lipase on chitosan.

The reader wishing to update his information on chitinases is referred to two recent books **(1,2)**.

When oligomers are sought, a way to circumvent the inherent difficulties of chitinases, enzymes that degrade chitin to the dimer, is to take advantage of the unspecific activity of chitinases towards chitosans **(3)**; if N-acetylated oligomers are sought, acetylation can be easily carried out. According to Aiba **(4)** variously acetylated chitosans are hydrolyzed with *Streptomyces griseus* chitinase.

Bacillus sp 7-M chitosanase is specific toward the GlcN-GlcN bonds in partially N-acetylated chitosan, and at least 3 GlcN residues are necessary for the hydrolysis of chitosan by chitosanase.

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Aiba and Muraki (5) have studied various enzymes, and expressed their preference for hemicellulase. For the preparative-scale production, chitosan is hydrolyzed by hemicellulase and acetylated with acetic anhydride.

The ability of lysozyme to depolymerize chitins and chitosans is well assessed in the literature; the reader is referred to a recent article on the subject (6).

Papain (EC# 3.4.4.10) is widely used in the food industry for tenderization of meat by limited hydrolysis of muscle protein and for a number of other purposes. Muzzarelli et al. (7) have reported on the ability of papain to depolymerize chitosans: a low-cost commercial enzyme, widely accepted in the food industry, could be a valid alternative to lysozyme and chitinases. The plant origin of the enzyme is a further appeal when certain sophisticated applications of chitosan hydrolysates are sought.

The enzymatic hydrolysis of chitosan promoted by papain has been followed by gel permeation chromatography. The profiles and positions of the curves are progressively altered during the course of the papain-promoted hydrolysis of chitosan. The chitosan fractions having the highest degrees of polymerization are depolymerized preferentially, and M_w , η , and R_G values are lowered. Viscometric measurements confirm that immobilized papain depolymerizes chitosan with high initial velocity, the more acetylated chitosan (0.42) being more susceptible to papain than chitosan (0.22) and chitosan (0.15). This holds true for chemically reacetylated chitosan (0.49) as well.

The behavior of papain in this respect is different from that of lysozyme which is more active on chitosan (0.22) than on more acetylated chitosans. The data suggest that papain acts on the link between glucosamine and N-acetylglucosamine units.

The unspecific hydrolytic action of 36 enzyme preparations including 5 lipase preparations (1 from porcine pancreas and 4 from fungi) on chitosan at pH 3.6 was reported by Pantaleone et al. (8). In lipases, proteases, cellulases, and hemicellulases a common lytic agent is not found; in fact, the pH and temperature optima profiles and the dependence on the degree of acetylation and the concentration of chitosan are different for the various enzyme preparations (9). Aiba and Muraki (5) have also shown that lipases are more convenient than chitinases for the preparation of oligomers.

Two lipases are used by Muzzarelli et al. (10,11), wheat germ lipase and a recombinant lipase. The latter is tested in order to verify if a recombinant enzyme is endowed with unspecific activity toward chitosan.

2—

Materials

1. Chitin and chitosan (practical grade from crab shells) and chitinase are from Sigma (St. Louis, MO) (*S. griseus*, C-1525, 500–2000 U/g solid), or from Anomeric, (Baton Rouge, LA) (recombinant chitinase from *Vibrio parahemolyticus*, C1010,

5 U/mg). Bio-Gel P-2, P-4 (200–400 mesh) are from Bio-Rad (Hercules, CA). Standards for TLC from GlcNAc to (GlcNAc)₄ are from Seikagaku (Rockville, MD), and (GlcNAc)₅₋₇ are prepared by acid hydrolysis (12). Fifty millimolar sodium phosphate buffer, pH 6.0, with 0.02% NaN₃; 5% BSA in sodium phosphate buffer; 20% trichloroacetic acid (TCA); 50% H₂SO₄; solvent for TLC:acetonitrile/H₂O(3:1).

2. Two chitosan samples from Katakura Chikkarin (Japan) are heated at 110°C in aq 47% alkali for 1 h under nitrogen. Reacetylated chitosans are prepared by N-acetylation under homogeneous conditions according to the method of Hirano et al. (13). Chitinases from *S. griseus* (0.86 U mg⁻¹, Sigma), *Bacillus sp* (0.045 U/mg, Wako, Japan), and *Bacillus sp* PI-7S (0.053 U/mg, Pias, Japan) are used without further purification. Authentic oligomers (*n* = 2–6) are from Seikagaku Kogyo (Japan).

3. Chitosanase is purified from the culture broth of *Bacillus sp* no. 7-M, and the active fraction is separated by column chromatography on CM-Sephadex C-50.

4. Hemicellulase is obtained from Amano Pharmaceuticals (Japan).

5. Hen egg white lysozyme is obtained from Seikagaku Kogyo.

6. Chitosan (0.23) (CTA-3 from *Chionoecetes opilio*) is supplied by Katakura Chikkarin (Tokyo, Japan); chitosan (0.15) from *C. japonicus*, is from the same supplier; shrimp chitosan (0.20) (Seacure 343) is supplied by Pronova (Drummond, Norway), and chitosan (0.42) from *Euphausia superba*, by Rybex (Gdynia, Poland).

Control chitosans are obtained from 7-d old (1.0%) lactate solution of these commercial chitosans; after dialysis against 0.1 M NaOH and then water, they are freeze-dried. Chitosans are reacetylated according to Hirano et al. (13).

Papain from *Carica papaya* by Calbiochem (La Jolla, CA) is immobilized on lobster chitin powder as previously described by Muzzarelli et al. (7). The immobilized enzyme is stored at 4°C in the wet state, and used for most experiments with no further treatment. A freeze-dried preparation is also used.

7. Chitosans are supplied by Katakura Chikkarin (from *C. opilio*), lot no.B-00123(6), average molecular weight 698 kDa, degree of acetylation 0.15; lot no. CTA-3 average molecular weight 537 kDa, degree of acetylation 0.23; Fluka low molecular weight 22741 ca. 70 kDa, medium molecular weight 22742 ca 750 kDa and high molecular weight 22743 kDa, >1 MDa; Pronova Seacure 343 ca. 400 kDa and degree of acetylation 0.18. Chitosans supplied by Daras (Marseille, France) (180 kDa), and by SIRC (Milano, Italy) are also used. Chitosan is dissolved in lactic acid solution (1.0%) and dialysed using Visking tubing with cutoff value 15,000 for 1 d, then freeze-dried. Chitosan lactate salt is used as a soluble substrate in lactate buffer solution at different pH values. Chitosan glycolate salt is also used.

Wheat germ lipase (EC# 3.1.1.3) is purchased from Sigma (lot no. H7155, 9.6 U/mg). A stock solution (5.0 mg/mL) is prepared and used for further dilutions. Recombinant lipase B from *Candida antarctica* and amylase from human saliva (EC# 3.2.1.1) are supplied by Fluka.

Table 1
Enzymatic Depolymerization of Chitin with Chitinases

Temperature, °C	Time, h	Products
4	24–36	(GlcNAc) _{1–6}
22	14–18	(GlcNAc) _{1–5}
37	8–10	(GlcNAc) _{1–4}
45	4–6	(GlcNAc) _{1–3}

3— Methods

3.1— *Chitin Depolymeration with Chitinases*

Chitin is swollen by treatment with acid to make it more easily digestible by chitinases. Crab chitin (10 g) is mixed with concentrated HCl (100 mL) and stirred slowly at 4°C for 24 h. The viscous material is diluted with cold water (1 L). The swollen chitin is recovered by filtration on a Büchner funnel with a glass microfiber filter. The white amorphous solid is neutralized with 2 N NaOH and washed extensively with water. The washed chitin is kept at 4°C.

3.1.1— Preparation of Chitooligomers

1. According to Zhu and Laine (**14**), a swollen chitin suspension (10 mL) is centrifuged at 5000 rpm for 10 min.
2. The white slurry (2 g) at the top layer is mixed with phosphate buffer, pH 6.0 (15 mL), containing 5 U chitinase. Enzymatic reaction is considered complete when the cloudy white chitin suspension becomes a clear solution. The reaction time, temperature and products are shown in **Table 1**.
3. After incubation, the mixture is heated at 100°C for 3 min to inactivate the enzyme. The yield of chitooligomers is 20–25 mg from 2 g chitin slurry.
4. Thanks to the unique stability of the chitinases from *Vibrio* and *Streptomyces* (**15**), a preparation of chitooligomers is also feasible as follows: The white chitin slurry (2 g) and chitinase (5 U) are mixed with 2 mL phosphate buffer, pH 6.0, and transferred into a dialysis bag (1.5 × 5 cm).
5. The bag is soaked in a centrifuge tube (50 mL) containing the same buffer (15 mL) and shaken constantly at 4–45°C.
6. When the solution inside the dialysis bag becomes clear, 2 g chitin slurry is added again and the bag soaked in 15 mL fresh phosphate buffer.
7. This procedure can be repeated at least five to eight times without significant loss of enzyme activity. Recovery of chito-oligomers from 10 such digestions is 150–200 mg.

3.1.2— Purification of Chitooligomers

1. A mixture of chitooligomers obtained by enzymatic hydrolysis is lyophilized, dissolved in a small volume of water, and applied to a Bio-Gel P-4 column. A

column size of 1×150 cm is suitable for purification of 1–10 mg of chito-oligomer mixture.

2. Chito-oligomers are eluted with water with a flow rate of ca. 5 mL/h, and fractions (1.5 mL each) are collected.

3. Fractions 38 to 57 from the Bio-Gel P-4 column are applied to a TLC plate.

4. The oligosaccharides are developed with the solvent acetonitrile/H₂O (3:1) and visualized by spraying the plate with 50% H₂SO₄ and heating at 120°C.

3.2—

Chitosan Depolymerization with Chitinase.

1. Chitosan ([0.13], 200 mg) is dissolved in 5 mL 1 M AcOH, aq 1% NaN₃, and 14.7 mL water, and the pH of the solution is adjusted to 5.0 with 3.5 mL 1 M NaOH.

2. Chitinase from *S. griseus* (1 mg/0.5 mL) is added and the mixture is incubated at 37°C for 7 d.

3. The mixture is concentrated to 8 mL and diluted with 24 mL methanol.

4. Acetic anhydride (1 mL) is added, the mixture is stirred at room temperature for 5 h, and 5 mL 1 M NaOH is added.

5. The mixture is centrifuged and the supernatant solution is concentrated to ca. 5 mL.

6. The precipitates are extracted with 20 mL water and the extract is combined with the concentrated supernatant.

7. The mixture is concentrated to 13 mL and poured onto a toyopearl HW-40S column (5×68 cm).

8. The column is eluted with degassed water at a flow rate of 94 mL h⁻¹.

9. Each fraction (3.13 mL) is analyzed by HPLC.

10. Five combined fractions are lyophilized and their purity is checked by HPLC. The oligosaccharides are separated into five peaks, corresponding to hexamer, pentamer, tetramer, trimer, and dimer of GlcNAc, respectively. The yields of dimer to hexamer are 1.9, 50.5, 50.9, 39.1, and 24.5 mg, respectively, after lyophilization. The purities of lyophilized oligosaccharides, calculated from the peak areas of HPLC patterns, are 97, 98, 94, 92, and 96% for dimer to hexamer, respectively.

3.3—

Hydrolysis of Chitosan by Chitosanase

1. The hydrolysis of chitosans with different degrees of acetylation is done as follows (**16**). A reaction mixture consisting of 60 mL 0.5% chitosan ([0.50], [0.30], [0.24], and [0.01]) in 0.1 M acetate buffer (pH 6.0) and 90 µL of chitosanase (5 U) is incubated at 37°C.

2. The reaction is stopped by boiling for 4 min at the time indicated in **Fig. 1**.

3. The increase of the reducing sugar is measured by the modified Schales method with GlcN as a standard.

4. The hydrolysis products are analyzed by HPLC.

5. The hydrolyzate of chitosan (0.24) is prepared as follows: A reaction mixture consisting of 200 mL 0.5% chitosan (0.24) in 0.1 M formate buffer, pH 6.0, and 0.9 mL of chitosanase (16.5 U) is incubated for 24 h at 37°C and the reaction is stopped by boiling for 4 min.

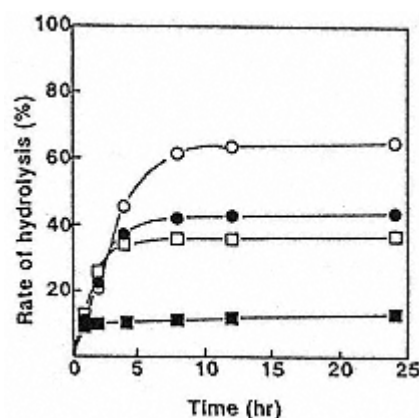


Fig. 1.

The hydrolysis of chitosans with different degrees of acetylation by *Bacillus* sp.No. 7-M chitosanase.

■-■, chitosan [0.50]; □-□, chitosan [0.30]; ●-●, chitosan[0.24]; ○-○ chitosan [0.01]. From Izume et al. (16).

6. The hydrolyzate is concentrated to about 1/20th using a rotary evaporator.

7. The yields of tri-, tetra-, penta-, and hexasaccharides of GlcNAc are 23.4, 14, 8.2, and 12%, respectively, on the basis of the weight of chitosan used.

3.4—

Hemicellulase

1. Chitosan (10 mg) is dissolved in 16 mL acetate buffer, pH 4.5, and the solution is mixed with the enzyme (20 mg) solution (0.4 mL).

2. The mixture is incubated for 11 d at 37°C.

3. The hydrolyzates are N-acetylated with acetic anhydride.

4. Higher oligomers are separated as described in Fig. 2. They are selectively precipitated from an aqueous solution by adding methanol. The solubility of (GlcNAc)_n decreases with increasing n.

5. The solubility is examined as follows: 5 mL methanol is added to 2 mL aqueous solution of pentamer (2.34 mg) and hexamer (4.64 mg). The pentamer remains in the solution (76%) while only 18% of hexamer remains. In addition, 96% of hexamer is precipitated from the solution when 2 mL methanol is added. Addition of three- or fourfold of methanol makes it possible to separate hexamer from lower oligosaccharides.

3.5—

Lysozyme

3.5.1—

Hydrolysis of Colloidal Chitin by Lysozyme (Method A)

A colloidal chitin suspension (0.5% w/v, 10 mL) in 0.2 M acetate buffer pH 5.4, with 0.05% NaN_3 is incubated with lysozyme (2 mg/mL, 1 mL) at 37°C for 3 d and then 1 mL lysozyme solution is added. After 3 d the suspension is centrifuged and the products in the supernatant solution are analyzed by HPLC.

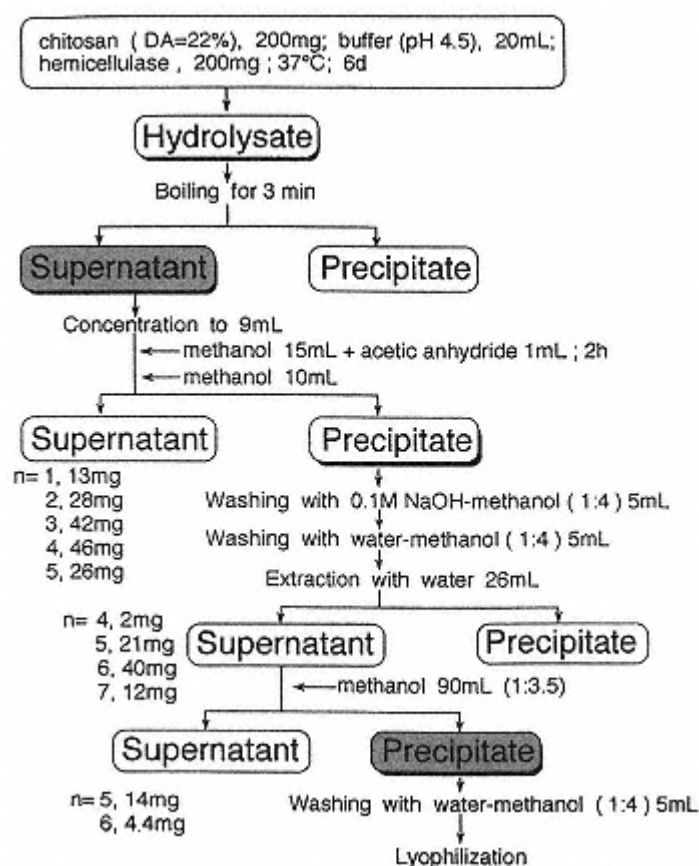


Fig. 2.

Process of preparation and separation of $(\text{GlcNAc})_n$. Final yield 51 mg.

3.5.2—

Method B (Large Amount of Lysozyme)

Reacetylated chitosan ([0.30–0.72], 100 mg) is dissolved as described above, to give a 0.5% solution. Lysozyme (20 mg) is added and the mixture is incubated at 37°C. At intervals, a portion (0.5 mL) is taken out, acetylated, and analyzed by HPLC.

3.5.3—

Separation of $(\text{GlcNAc})_n$ by Gel Filtration Chromatography

1. Reacetylated chitosan ([0.30–0.72], 100 mg) is dissolved in 1 M AcOH (4 mL), 1 mL 1% NaN_3 , and 11.6 mL water, and the pH of the solution is adjusted to 5.4 by 1 M NaOH (3.4 mL).
2. Lysozyme (20 mg) is added and the mixture is incubated at 37°C for 7 d.
3. The mixture is concentrated to 7 mL and diluted with 28 mL methanol, and 0.5 mL acetic anhydride is added.

4. The mixture is stirred at room temperature for 2 h and then 1 M NaOH (3 mL) is added.
5. The solution is centrifuged and the supernatant solution is concentrated to 1 mL.
6. The precipitate is extracted with 20 mL water, and the extract is combined with the concentrated supernatant.
7. The mixture is concentrated again to 17 mL and put onto a toyopearl HW-40S column (5 × 70 cm) previously equilibrated with water.
8. The column is eluted with degassed water at a flow rate of 91.6 mL h⁻¹.
9. Each fraction (4.6 mL) is analyzed by HPLC.
10. Four combined fractions are lyophilized and the purity is checked by HPLC.

3.5.4—

Analysis of Hydrolysates by HPLC

Chitooligomers are analyzed by two HPLC sets. The first set comprises a Tosoh TSKgel Amide-80 column (4.6 × 250 mm; flow rate, 1.0 mL/min; eluent, 65:35 MeCN-water). The second set comprises an Asahipak NH2P-50 column (4.6 × 250 mm; flow rate, 1.0 mL/min; eluent, 70:30 MeCN-water). Quantitative analysis is done using an authentic mixture of oligomers, and a UV detector at 210 nm.

N-Acetylation of the hydrolysates doubled the amount of total (GlcNAc)_n and (GlcNAc)₃, and increased the amount of (GlcNAc)₄ ninefold. The amount of (GlcNAc)_n after hydrolysis and N-acetylation increased with the increase of degree of acetylation of partially N-acetylated chitosans (PNACs). The optimum degree of acetylation for the formation of oligomers is ca. 70%. The yields of (GlcNAc)_n ($n = 2 - 5$) after gel filtration chromatography are 16.3, 10.3, 18.0, and 1.7 mg, respectively, from the hydrolysates of 72% N-acetylated chitosan (100 mg). This procedure is useful for the production of (GlcNAc)₂, (GlcNAc)₃, and (GlcNAc)₄.

Figure 3 shows the effect of the concentration of acetic acid and methanol on degree of acetylation. The chitosan concentration is 0.3% and the molar ratio of acetic anhydride to amino groups is 0.60 (**17**).

Partially reacylated chitosans are suitable for hydrolysis with lysozyme. The recommended concentrations for acetylation are: chitosan 1%, acetic acid 0.4%, methanol 50%.

The highest digestibility of chitosans is due to the blocks of NAcGlc sequences. The hexamers containing three or more acetylated units contribute mostly to the initial degradation velocity. This information is based on the Michaelis-Menten analysis of the degradation data for various hexamers under the action of lysozyme (**18**). The substrate specificities of human and hen egg white lysozymes with respect to partially N-acetylated chitosans are undistinguishable. Tailor-made chitosans with a predetermined degradation rate in the human body can be made by simply controlling their degree of acetylation (**19**).

Lysozyme does not seem to lead straightforwardly to the production of oligomers: partially N-acetylated chitosans (N-acetyl-N-hexanoyl) subjected to the

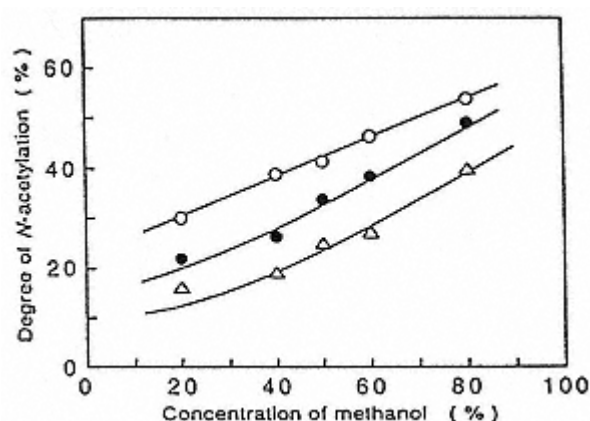


Fig. 3.
Effect of the concentration of methanol and acetic acid on the degree of acetylation: acetic acid, 0.4% (○), 1% (●), and 3% (△); molar ratio of acetic anhydride to amino group, 0.605; chitosan, 0.3%.
From Aiba (17).

action of lysozyme show final average molecular weights of $1\text{--}10 \times 10^4$ over a contact period of 24 h (20).

3.6—

Immobilized Papain

Depolymerization is carried out in 1% lactic acid at 20°C in the presence of immobilized papain: at fixed times, aliquots are taken, filtered on filter paper to remove the immobilized enzyme and diluted (10–20 fold) with aqueous solutions of acetic acid and sodium acetate, in such a way as to prepare solutions for η GPC, and LS measurements of the following composition: 0.5 M acetic acid, 0.2 M sodium acetate, and 1.0 g/L lactic acid.

The elemental analysis and the molecular parameters for the control chitosan (0.23) and for the same contacted with immobilized papain for 7 d and isolated as the control sample are found to be the following: Chitosan (0.23): N 7.42, C 40.77, H 6.91, N/C 0.182; η 11.77 dl/g, M_w 780,000, A_2 2.10×10^{-3} , R_g 1190 Å. Papain-treated chitosan (0.23) (7 d): N 7.31, C 40.11, H 6.69, N/C 0.182, η 3.66, M_w 183,000, A_2 3.10×10^{-3} , R_g 700 Å.

3.7—

Lipases.

The reaction is followed using a viscometer (Haake Rotovisco RV-20, M5) driven by a personal computer with Haake Rotation software. The doublewalled NV rotor is housed in an 11-mL cup filled with the solution under study (10 mL), to which 1 mL of enzyme solution is added with the aid of a syringe after reaching the desired temperature and just before starting measurements. The measurements are done at a shear rate value (200 s^{-1}) which does not gen-

erate excessive mechanical degradation. Such shear rate is reached in 0.1 min and protracted for 9.8 min with 0.1 min deceleration to 0. Three hundred eightyfour readings are automatically recorded in the 10 min period. The initial 0.1 min during which the rotor accelerated and mixed the solutions is not taken into consideration when calculating the initial velocity. Initial velocities (mPa.s.min^{-1}) are read graphically from the points plotted in the subsequent 0.3-min period. The overall error involved is within 5%. Measurements are also done on controls, where the lipase solution is replaced with water. In general, the final enzyme concentration is 0.45 mg/mL, unless otherwise stated. The viscosity decrease percent (VDP) is calculated as $\text{VDP} = (V_0 - V_t) / V_0$, where V_0 is the initial viscosity and V_t is the viscosity after 10 min of incubation with lipase preparation in the viscometer. The slight viscosity decrease recorded for the control is taken into account. The initial velocity for the enzymatic reaction is expressed in terms of mPa.s.min_i , and is the slope of the VDP vs time curve in the time interval 0.1–0.4 min. Other measurements (intrinsic viscosity and gel permeation chromatography) are performed as described by Terbojevich et al. (21).

The plot of viscosity vs time for the chitosan lactate salt solution (10 g/L) containing wheat germ lipase preparation at pH 5.8 and 25°C indicates that the hydrolytic reaction is fast and still in progress after the 10 min measurement time period. Three curves are presented in **Fig. 4** for the following lipase concentrations: 4.5, 45, and 450 mg/L. Measurements taken in this range showed a logarithmic dependence of V_i on the enzyme concentration. When the data are replotted in the double logarithmic presentation for three temperatures, curves slightly bent downward are obtained, indicative of deviation from usual kinetic models, and confirming the progress of the reaction beyond the time selected for the experiment.

The effect observed after 10 min treatment in terms of VDP is in the range of 50–60% for lipase concentration 0.45 g/L. Therefore the lipase concentration 0.45 g/L is preferred.

When the recombinant lipase is used, the unspecific activity is present and leads to measurable viscosity reduction, but the depolymerization of chitosan is sluggish compared to the wheat-germ lipase. In fact, the viscosity of the chitosan solutions is reduced to one half only after one d rather than within minutes, as it is the case for wheat germ lipase. The recombinant lipase can be activated by CaCl_2 when the substrate is chitosan; it is reversibly inhibited by high acetate concentrations, and keeps its activity for days at 50°C.

The gel permeation chromatography curves indicate that the recombinant lipase preferentially acts on chains with high degree of polymerization, because the corresponding fractions are progressively disappearing with time (left hand side part of the plot in **Fig. 5**), while the average molecular weight values

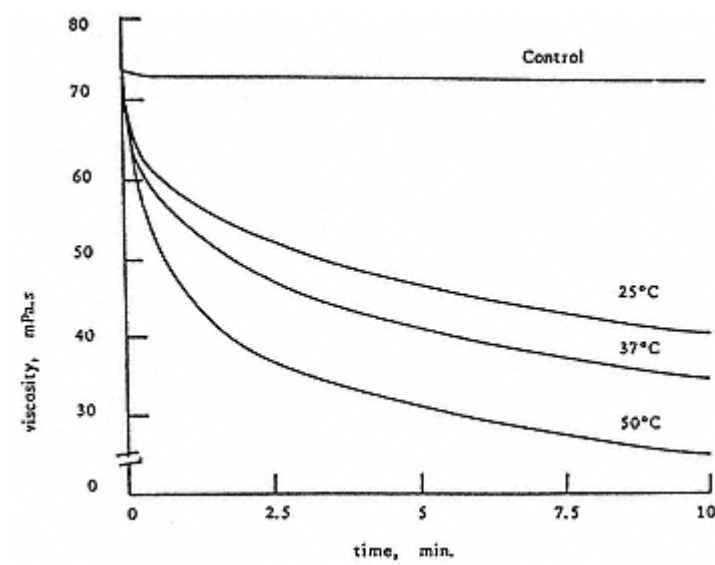


Fig. 4.

Variation of the intrinsic viscosity vs time, for chitosan lactate in the presence of wheat germ lipase at three concentrations: 4.5, 45, and 450 mg/mL. Chitosan lactate concentration 10 g/L; pH 5.4. From Muzzarelli et al. (11).

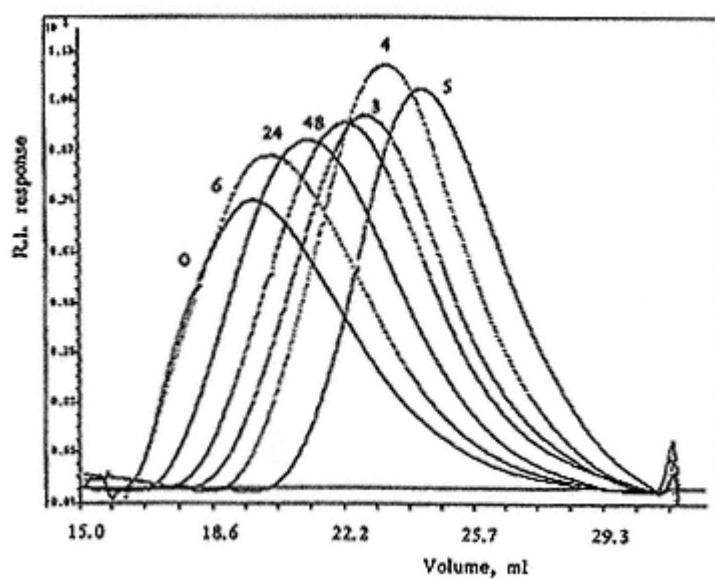


Fig. 5.

Gel permeation chromatography curves for the depolymerization of Katakura CTA-3 chitosan under the action of recombinant lipase, after various contact times: 0, 6, 24, 48 h and 3, 4, 5 d. From Muzzarelli et al. (11).

move towards lower values and, the Mw/Mn ratio decreases from 4.03 to 2.54 in the 5-d period. These enzymatic reactions do not obey a simple kinetic model and, of course, the Lineweaver-Burk plot is impossible to draw.

4—

Notes

1. The major product of the chitin depolymerization reaction promoted by the chitinase is $(\text{GlcNAc})_2$ and the accompanying oligomers are from GlcNAc to $(\text{GlcNAc})_6$. The dimer prevails in spite of the precautions taken (simultaneous dialysis and low temperature).

2. Chitosan depolymerization with chitinase has two advantages: The chitosan to be used can easily be obtained from crab shell chitin by N-deacetylation under heterogeneous conditions and a highly deacetylated sample is unnecessary. Secondly, chitinase is commercially available. The amount of chitinase recommended is 1 mg for 200 mg of chitosan. Nevertheless chitinases do not seem to be the most convenient enzymes if oligosaccharide production is sought. They have inherent tendency to produce the dimer as a final sole product. Moreover chitinases of different origins have different mechanisms of action (22). Finally, the chitin itself as a substrate for chitinases may present problems. The entire preparation of the chitin should be known if a serious assessment of its suitability has to be made. Depending on the preparation mode, chitins might be only partially accessible to chitinases, even to those that are secreted in molting fluids for the rapid degradation of solid chitin, because the native structure of chitin has been lost due to bleaching and similar treatments including incorrect use of NaOH. Attention has been directed to these aspects by Bade (23).

3. The hydrolysis of chitosan by chitosanase leads to results similar to those obtained with chitinases. The digestibility of various chitins by the chitinase from *Bacillus sp* PI-7S is much higher than that by lysozyme, and β -chitin is digested more smoothly than α -chitin.

4. The yield of hexamer is about 18% when using hemicellulase preparation. The separation process is simple and does not include a chromatographic step. Cellulases are used for the same purpose by Muraki et al. (24).

5. Chitin deacetylated under homogeneous conditions is hydrolyzed by lysozyme more rapidly than that deacetylated under heterogeneous conditions. Chitins from shrimp shell and squid pen show the same degree of digestibility by lysozyme in spite of a difference in the crystal structure of the original chitins. The crystal structure of chitin and the degree of N-acetyl group aggregation affect the enzymatic digestibility of chitins and deacetylated chitins. The hydrolytic activity of lysozyme toward chitins and chitosans should be considered unspecific since lysozyme is a muramidase. The difficulty of using lysozyme for the preparation of oligomers is partially circumvented by chemical derivatization, such as the production of glycol chitin, methylpyrrolidinone chitosan and reacylated chitosans, that are water-soluble at the pH values of maximum enzyme activity.

At 37°C and optimum lysozyme pH value, the K_m value for glycolchitin is 2.95 kg/m³ (lysozyme 0.012 g/L) (25); for MP chitosan is 71 kg/m³ (lysozyme 0.4 g/L) (26) while Aiba (27) indicates much lower values (in the range of 0.05–0.5 kg/m³) for chitosans and reacetylated chitosans. Nevertheless he points out that the initial velocity data might be not indicative of the real susceptibility of a given chitosan because the hydrolysis rates change with the progress of the reaction, depending on degree of acetylation and acetyl group distribution: actually the molecular weight observed after various days of contact are in the range of 95–130 kDa.

6. Chitosans are promptly depolymerized by papain, which acts preferably on the longest chains at room temperature. Papain conveniently immobilized on chitin powder can be retrieved from the chitosan solution for further use.

Compared to chemical hydrolysis, the method proposed offers a number of advantages, mainly slightly acidic pH values and 20°C rather than warm 0.6 M HCl solutions. For instance, the chitosan (0.42) undergoes with papain an intrinsic viscosity drop of 66% within 48 h at 25°C but with 0.6 M HCl at 25°C it would take 20 d to reach the same result (21).

The hydrolytic action of papain lowers the average molecular weight of chitosan of at least one order of magnitude, and therefore provides low molecular-weight chitosans that are especially sought for use in the medical and pharmaceutical areas.

7. All of the chitosans tested are highly susceptible to depolymerization by wheat germ lipase and, to a lesser extent, by recombinant lipase from *Candida antarctica*. Immobilized lipase is particularly attractive for technical purposes, and the data obtained using recombinant lipase have fundamental importance because they definitely rule out the action of unidentified “impurities” in the enzyme preparations. All of the lipases tested in various laboratories depolymerize chitosan, it seems therefore reasonable to expect that human lipases are active on chitosan as well. This conclusion has far-reaching implications in view of the role of human lipases in various fluids (i.e., plasma, saliva, and gastric and pancreatic juices) when chitosans are used (i.e., wound dressings, and dietary foods).

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17—

Synthesis of Homo- and Hetero-Oligosaccharides from Underivatized Sugars Using Glycosidases

Christopher Bucke, John Packwood, Sony Suwasono, and Robert A. Rastall

1—

Introduction

Progress in the development of knowledge of “glycobiology” is seriously impeded by the lack of quantities of characterized oligosaccharides available at reasonable prices. Specialist companies offer chemically synthesized materials, but prices tend to be high because of the complexity of the synthetic methods. Oligosaccharides may be isolated from natural sources but, again, prices are high. An alternative approach to oligosaccharide synthesis is to use glycosidases to catalyze the condensation of underivatized sugars in conditions of low water activity. This approach has the instant attraction that both enzymes and substrates are inexpensive and readily available: The obvious drawback is that, at equilibrium, the amounts of uncondensed substrates in the reaction mixture will exceed the amounts of oligosaccharide product(s) so isolation of the product(s) may not be straightforward. The history of oligosaccharide production is reviewed by Rastall and Bucke **(1)**. Serious studies commenced in the late 1980s by Hedbys et al. **(2)** and Johansson et al. **(3)**. These showed that condensation reactions retained the anomeric specificity of the enzymes used but that there was little regiospecificity of synthesis, so that equilibrium reaction mixtures contained several different oligosaccharides. For instance, at equilibrium, the reaction mixture of D-mannose incubated with jack bean α -mannosidase contained at least the 1,6-, 1,3-, 1,2-, and 1 – 1 linked mannobioses, with the 1,6-linked disaccharide predominating **(3)**. Rastall et al. **(4)** demonstrated the presence of the 1,4-linked disaccharide in similar reaction mixtures. Hedbys et al. **(2)** made the important observation that, if enzymes with different substrate selectivity were available, purification of the desired

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product could be aided by the selective removal of unwanted products from the reactions mixtures. There is now an extensive body of literature indicating that all glycosidases may be used to synthesize homo-oligosaccharides and that most glycosidases catalyze the synthesis of hetero-oligosaccharides (5–8). Generally, oligosaccharide synthesis shows the lack of regioselectivity observed with jack bean α -mannosidase but Suwasono and Rastall (9) have demonstrated the highly regioselective synthesis of α -1,2-linked manno-oligosaccharides using α -mannosidases from *Aspergillus* species.

The majority of those studying oligosaccharide synthesis using this method have aimed for high yields of product by allowing the reactions to reach equilibrium. A few studies (e.g., refs. 10 and 11) have followed the progress of condensation reactions aimed at producing hetero-oligosaccharides. Patikis and Bucke (10) showed that the initial synthetic product detected when yeast α -glycosidase was incubated with a mixture of D-glucose and L-fucose was isomaltose: other oligosaccharides, homo- and hetero-, accumulated with time, some of them appearing transiently in the reaction mixture. Svasti et al. (11) showed that the initial, kinetically favored product of the incubation of *Albizzia procera* α -mannosidase with D-mannose alone or with a mixture of D-mannose and raffinose was α -1,4-mannobiose: this remained a minor component of the reaction mixture as other oligosaccharides accumulated and plausibly acts as the donor substrate in the later reactions. The regioselectivity of hetero-oligosaccharide synthesis is unpredictable: Svasti et al. (11) demonstrated that plant α -mannosidases catalyze the condensation of mannose with the galactose, fructose and possibly glucose residues of raffinose, Suwasono and Rastall (12) showed that the α -mannosidase from *Aspergillus phoenicis*, which shows selectivity for the synthesis of 1,2 links when provided with mannose alone synthesized products with α -1,6, α -1,3 and α -1,1- α linkages when provided with maltose, lactose, and isomaltulose, respectively.

So, glycosidase-catalyzed condensation reactions provide means of synthesizing a very wide range of oligosaccharides, some of which do not occur in nature. The synthetic procedure is straightforward, analysis of the products and isolation of individual oligosaccharides from the reaction mixtures presents more problems. HPLC is essential to detect the synthesis of products: Most stationary phases can separate oligosaccharides of different sizes but cannot separate individual oligosaccharides with the same degree of polymerization. High-performance anion-exchange chromatography (HPAEC) does allow the separation of individual oligosaccharides but different gradients of eluant may be required to quantify all the components of a reaction mixture. True quantitation of products is difficult as pure standard materials are rarely available.

This chapter describes a general procedure for oligosaccharide synthesis.

2—

Materials

1. Sugars: Many sugars are available at high standards of purity from the main chemical suppliers. Many rarer oligosaccharides, such as mannobioses and mannotrioses, are available from Dextra Laboratories (Reading UK).
2. Glycosidases: Many glycosidases are available (from Sigma) that indicate the presence of enzyme activities present in addition to the enzyme purchased. These are not normally a problem. The major commercial enzyme producers, for example, Novo-Nordisk (Bagsvaerd, Copenhagen, Denmark), are prepared to provide samples of enzyme preparations free of charge to researchers. Most such enzyme samples may contain many different glycosidase activities.
3. 0.01 *M* Sodium acetate/acetic acid buffer, pH 5.0.
4. Bio-Gel P-2 fine grade, particle size 45–90 μm (Bio-Rad).
5. Econo-columns (2.5 $\times$ 120 cm) (Bio-Rad).
6. Sodium hydroxide (50% w/v).
7. HPLC grade acetonitrile (Rathburn, Walkerburn, Scotland, UK).
8. HPLC grade water.
9. 0.45- μm Membranes (Millipore).
10. HPLC columns: e.g., Aminex HPX-42A (Bio-Rad), C_{18} column (Hichrom, Reading, UK), CarboPac PA1 (Dionex UK; Camberley, Surrey, UK).
11. Refractive index detector plus integrator, e.g., Hewlett Packard HP 3996A. For HPAEC a Dionex PAD II pulsed amperometric detector with a gold electrode is ideal.
12. GC-MS system equipped with, e.g., a BPX-5 capillary column (0.32 mm $\times$ 50 m) (SGE, Milton Keynes, UK).
13. Dimethylsulfoxide.
14. Methyl iodide.
15. Chloroform.
16. Anhydrous sodium sulfate.
17. Trifluoroacetic acid (5 *M*).
18. Nitrogen.
19. Sodium borohydride reagent: 0.5 *M* sodium borohydride in 2 *M* ammonia.
20. Acetone.
21. 18 *M* Acetic acid.
22. Ethyl acetate.

23. Acetic anhydride.
24. Perchloric acid (70%).
25. 1-Methylimidazole.
26. Dichloromethane.
27. High-purity carbon, e.g., Darco G60, 100 mesh (Sigma).
28. Celite 535 (Fluka, Buchs, Switzerland).
29. Clean sand.
30. Ethanol.
31. Chromatography column, e.g., 5 cm diameter, 50 cm high.

3—

Methods

3.1—

Oligosaccharide Synthesis

1. Weigh out the desired quantity of substrate (see **Note 1**).
2. Dissolve the appropriate amount of enzyme to give a final activity of about 1 International Unit per gram of reaction mixture at 70% w/w final sugar concentration in 0.01 M acetic acid/sodium acetate buffer (see **Note 2**).
3. Mix the enzyme solution with the sugar. At 70% (w/w), the consistency of the final mixture will be a viscous paste.
4. Incubate at 60°C for up to 7 d.
5. Heat the reaction mixture at 100°C for 5 min to inactivate the enzyme. This is ESSENTIAL as enzymes retain their full activity in the very concentrated sugar solutions used and would hydrolyze the oligosaccharide products rapidly on dilution of the reaction mixture if not inactivated.

3.2—

Product Analysis.

1. Inject 10-μL aliquots of reaction mixtures into the HPLC column selected. Satisfactory results are obtained using a 3-μ Spherisorb amino column (4.6 × 150 mm) eluted isocratically using 80% (w/v) acetonitrile in water at a flow rate of 1.5 mL/min.
2. If HPAEC is used dilute 20 μL of the sample 500 fold with HPLC grade water. Filter through a 0.45 μ membrane (Millipore) before injection. Elute with 0.05 M sodium hydroxide (prepared by adding 2.6 mL of 50% [w/v] sodium hydroxide to degassed HPLC grade water to give a final volume of 1 L).
3. If standard materials are unavailable, calculate quantities of products on the basis of peak areas.

3.3—

Partial Purification of Oligosaccharides

1. Oligosaccharides may be separated from monosaccharides and from each other on the basis of size using a column of Bio-gel P-2 fine grade using degassed deionized water as eluant. For a column 2.5 cm × 120 cm use a flow rate of 0.5 mL/min. (see **Notes 3** and **4**). Analyze fractions using an R. I. detector.
2. Pool fractions containing products and freeze-dry prior to structural analysis.

3.4—

Separation of Oligosaccharides Using Carbon-Celite Chromatography

Carbon has been used for the resolution of sugar mixtures into mono-, di-, and oligosaccharides for some decades but, despite the age of the technique, it is still useful and is often the method of choice for preparative-scale purifications, mainly because it is able to handle much larger quantities of material than most HPLC or gel-filtration methods. Although carbon is the absorbent, chromatography columns are usually mixed beds with either Celite or a coarse grade of carbon added to provide mechanical support and to prevent compression of the column, which would be detrimental to eluent flow rates.

1. Dry mix carbon (Darco G60, 100 mesh) and Celite 535, 1:1 by weight, then slurry in distilled water.
2. After thorough mixing, allow the preparation to settle and decant the supernatant to remove fines.
3. Prepare a glass chromatography column, e.g., 5 cm diameter by 50 cm, with a glass wool plug or coarse glass sinter covered with a layer of sand 1–2 cm deep.
4. Resuspend the carbon-Celite sediment in water and pour the slurry into the column.
5. Flow water through the column under gravity to pack the bed thoroughly.
6. When the column is packed, apply another layer of sand to the top surface. To pack a column of the dimensions described, 180 g each of carbon and Celite are needed.
7. Sample can be applied to the column at a loading of 1 g per 150 mL of column bed, usually at a concentration of 10% (w/v) total sugar. Filter through a 0.45- μ membrane filter to remove particulates.
8. Apply the sample to the column under gravity.
9. Elute with an increasing concentration of ethanol from 0 to 25% in water as a linear gradient or in 5% steps. Typically monosaccharides are eluted with pure water, disaccharides with 5% ethanol, trisaccharides with 10% ethanol, and so on. If a gradient is used the solvent flow is best provided using a peristaltic pump connected to a standard gradient former. Stepwise elution may be arranged using a pump or the “flash” chromatography method, which uses gas pressure.
10. Fractions collected may be tested for saccharide content using the phenol-sulfuric method or by TLC.

3.5—

Structural Analysis

Structural analysis using methylation, hydrolysis, and reduction followed by GLC analysis of the products allows the determination of the structures of the individual components of mixtures of oligosaccharides.

1. Dissolve 4–5 mg of oligosaccharide in dimethylsulfoxide (0.3–0.5 mL).
2. Add 20 mg of finely powdered sodium hydroxide and 0.1 mL of methyl iodide.
3. Vortex mix in a closed vial for 5 min.
4. Leave at room temperature for 1 h.
5. Add water (1 mL) and chloroform (1 mL); vortex mix.
6. Remove the water phase using a Pasteur pipet; discard.
7. Wash the chloroform layer five times with water.
8. After the final wash, remove any remaining water by the addition of anhydrous sodium sulfate.
9. Evaporate to dryness in a stream of nitrogen.

10. Add 0.3 mL of 5 *M* trifluoroacetic acid; heat at 100°C for 15 h in a sealed vial.
11. Cool and evaporate to dryness in a stream of nitrogen.
12. Add 1 mL of 0.5 *M* sodium borohydride in 2 *M* ammonia to the dry sample.
13. Heat at 60°C for 1 h; add acetone (0.5 mL) to stop the reaction.

14. Evaporate to dryness in a stream of nitrogen.
15. Dissolve the dry sample (now methylated, hydrolyzed and reduced) in 2 mL of 18M acetic acid.
16. Add 1 mL of ethyl acetate, 3 mL of acetic anhydride, and 0.1 mL of 70% perchloric acid. Mix and allow to stand for 5 min; cool on ice.
17. Add 5 mL of water, then 0.2 mL of 1-methylimidazole, stand for 5 min.
18. Add 1 mL dichloromethane; vortex mix. Stand to allow phases to separate.
19. Remove the organic phase; store this in a sealed vial prior to analysis by gas chromatography/mass spectrometry.
20. Satisfactory results are obtained using a gas chromatograph equipped with a splitless injector, a flame ionization detector, and a BPX-5 capillary column (5% phenyl equivalent modified siloxane, nonpolar) (0.32 mm × 50.0 m). Heat from 100 to 250°C at 3°C/min. Calibrate the MS detector from 33 to 400 amu with a scan rate of 1.9 scan/s. Record spectra at an electron multiplier voltage of 1800 with an ionization current of 50 mA and an ion-source temperature of 174°C.
21. Compare mass spectra with literature results (**13,14**).

4—

Notes

1. It should not be forgotten that some sugars crystallize with water of crystallization.
2. The quantity of enzyme used is not critical. High concentrations of sugar stabilize the enzymes against thermal inactivation, so it is reasonable to assume that the time taken for equilibrium to be reached will be inversely proportional to the enzyme activity used. Lower sugar concentrations than 70% (w/w) may be used: they are easier to handle but will give lower yields of product overall.
3. Some ingenious methods have been developed for removing unwanted components of reaction mixtures prior to chromatography. Smith (**15**) used pellets of the fungus *Ganoderma aplanatum* to ferment away mannose before purifying mannobioses on a column of Bio-Gel P2.
4. Recently, Svasti et al. (**11**) have achieved very satisfactory separations of individual disaccharides, trisaccharides and even tetrasaccharides, using a column of activated carbon (Bio-Rad).

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18—

Use of Fluorophore-Assisted Carbohydrate Electrophoresis (FACE®) in the Elucidation of N-Linked Oligosaccharide Structures

Harish P. M. Kumar, Patricia A. Denny, and Paul C. Denny

1—

Introduction

Fluorophore-assisted carbohydrate electrophoresis (FACE®) is a technique that has been successfully used to determine the structure of N-linked oligosaccharides (**1,2**). The method was originally introduced by Peter Jackson (**3**) to separate both mono- and oligosaccharides, and several reviews have appeared since then on this subject (**4–7**). The techniques used here are relatively simple and can be carried out in most biochemical laboratories.

The purpose of this chapter is to provide a detailed step-by-step procedure, based on the authors' experiences, for a novice user to be able to use this simple, but powerful technique to determine the N-linked oligosaccharide structures present in glycoproteins. Readers are directed to other reviews on the subject for additional information (**3–8**). A comprehensive volume on other methods of glycoprotein analysis has been previously presented by Hounsell(**9**) in the Methods in Molecular Biology series. Oligosaccharides are generally linked to glycoproteins through an asparagine (N-linked) or a Ser/Thr (O-linked) residue (**10**). Although the FACE technique may be used to determine the structure of other oligosaccharides (like the O-linked, and so forth), we are confining ourselves to the N-linked structures only to help understand the systematic approaches necessary for a successful determination of the oligosaccharide structures. In addition, whenever possible, commercially available reagents are used throughout to focus more on the methods and strategies associated with this technique.

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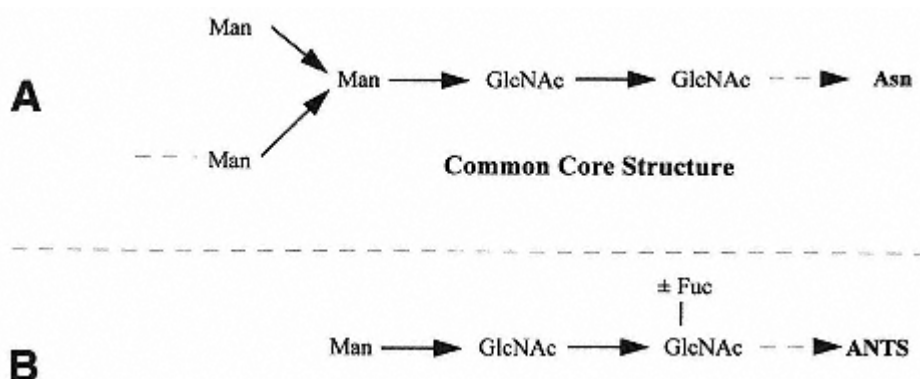


Fig. 1.

The tri-mannosyl core common to all *N*-linked oligosaccharides (A). The ANTS-labeled core standards used in the FACE technique is shown for comparison (B).

The *N*-linked oligosaccharides have a common core structure (**Fig. 1**), generally referred to as the tri-mannosyl core. There are three types of *N*-linked structures recognized, the high-mannose, complex, and hybrid structures. All these types have the common core as illustrated in **Fig. 2**. Additions to the core like the core fucosylation at the reducing *N*-acetylglucosamine (GlcNAc) end and GlcNAc on the mannose are common. Additional variations of these structures include addition of up to four (or five) chains to the tri-mannosyl core. The number of chains attached to the mannosyl core determines the antennary structure, and thus they are termed bi-, tri-, or tetra-antennary structures (**Fig. 3**). The position of attachment (like the 1–4, 2–3, and so on) between the carbohydrate units and the anomeric (α or β) configurations have been purposely omitted to simplify presentation of the structures. Anomeric nature and linkage positions are determined by the specificity of the exoglycosidases used in sequence determination.

The FACE technology involves two major procedures in the determination of the *N*-linked oligosaccharide structures. First, oligosaccharides are released from the glycoprotein using endoglycosidases. The most frequently used enzymes for this purpose are the endoglycosidase-H (endo-H; EC 3.2.1.96) and peptide-*N*-(*N*-acetyl- β -glucosaminyl)asparagine amidase (PNGase F; EC 3.5.1.52). The released oligosaccharides are then labeled with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonate (ANTS). The labeled oligosaccharides are separated by electrophoresis on precast polyacrylamide gels followed by isolation of the individual fluorescent bands while viewing under a UV lamp.

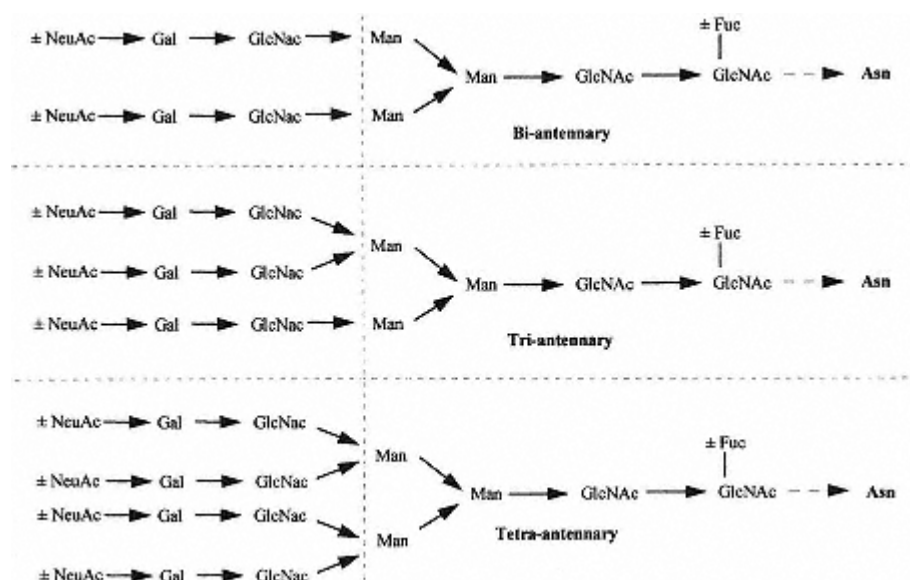


Fig. 2.

Three types of N-linked oligosaccharides commonly found in glycoproteins. The tri-mannosyl core common to all three types is seen to the right of the vertical line.

The second procedure involves the treatment of the isolated band(s) with a series of exoglycosidases followed by re-electrophoresis on polyacrylamide gels and then imaging the band patterns in a fluorescent imager. The authors have used the commercially available FACE imager and software extensively in these studies. The use of an imager is not required to complete oligosaccharide sequencing studies by this approach (2). However, an imager of the type noted above is recommended, because it greatly improves the accuracy, sensitivity, linearity, and speed of analysis and interpretation.

The mobility of all bands is measured in the gels relative to an ANTS-labeled glucose ladder consisting of polymers of glucose from 2 to 15 (or more) glucose units. Thus, it is necessary to include this glucose ladder in a lane on every gel run. The mobility of the glucose polymers is inversely proportional to the size of the polymer. However, the migrating distance is measured in terms of the glucose polymer size in units of degree of polymerization (DP). Thus maltose (two glucose units) has a DP of 2, maltotetraose (four glucose units) has a DP of 4, heptose has a DP of 7, and so on. Researchers using the FACE imager and software will be able to obtain these values as part of the imaging and analyzing protocol. The change in the DP values (Δ DP) assumes great importance particularly after treatment of an oligosaccharide with an exoglycosidase. The numerical value of Δ DP helps in determining the number

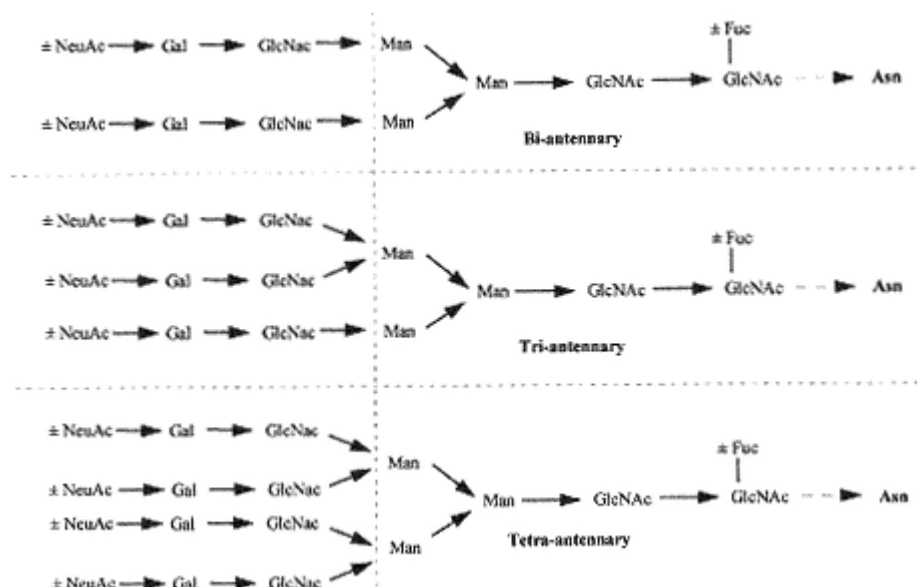


Fig. 3.

Antennary structures of *N*-linked oligosaccharides (complex). The number of chains attached to the (tri-mannosyl) core determines the antennary structure like the bi-, tri- or tetra-antennary oligosaccharide.

Table 1
Change in DP Value Owing to Loss of Monosaccharide Units
by Treatment with Exoglycosidases^a

Average Δ DP Shift/Monosaccharide	Monosaccharide removed
1	NeuAc
1	Gal
0.75	GlcNAc
0.75	Man

^aAdapted from refs (1) and (7).

of monosaccharide units removed and thus has a bearing on the number of branches of the oligosaccharide. The relative shift in mobility (Δ DP) of various monosaccharides is shown in **Table 1**. In addition, by including a quantitative standard in one lane of the gel, such as maltotetraose (G4), the amount of all the oligosaccharides in the gel can be determined. The sensitivity of the method is in the low picomole range. We will illustrate here the use of the FACE

technique to determine the sequence of selected oligosaccharides isolated from recombinant human factor VIII, and from mouse submandibular and sublingual gland mucin.

Obviously there are many potential applications of the FACE technique. The realization that oligosaccharide structures can be determined by a simple polyacrylamide electrophoresis technique has created an enormous interest for researchers in various areas. We can certainly expect an explosion in the use of this technique for oligosaccharide quantitation and structure analysis.

2—

Materials

2.1—

Equipment.

1. SE 2000 FACE workstation, that includes an imager, interfaced to an IBM® compatible Pentium computer with SE2000 Enhanced FACE Analytical Software. The FACE Imager contains a UV source and a CCD camera capable of capturing a fluorescent image of the gel band patterns (Glyko, Inc., Novato, CA).
2. Long UV transilluminator light box, model TL-33E (UVP, Upland, CA).
3. Electrophoresis gel box with high-efficiency recirculating cooling chambers (Glyko, Inc.).
4. Recirculating cooling device capable of cooling to 2–4° C type NESLAB RTE 134A or similar (Neslab, Union City, CA).
5. Electrophoresis power supply capable of delivering up to 3000 V. DC: IBI Model MBP 3000EP or equivalent (IBI, Ltd., Cambridge, England, UK).
6. Flat gel-loading micropipet tips: 0.1–200 mL capacity for loading samples on to gels.
7. Microtest tubes, 1.5 mL with O-ring screw caps or equivalent (Bio-Rad, Hercules, CA).
8. Thermometer to monitor the electrophoresis buffer temperature.
9. Slide-A-Lyzer 10K dialysis cassettes or equivalent to dialyze small volume of glycoprotein samples (Pierce Chemical Co., Rockford, IL).
10. Single-edge razor blade.

2.2—

Chemicals and Enzymes

1. FACE *N*-linked oligosaccharide profiling/sequencing gels: These gels are specially formulated between two optically clear glass plates for easy imaging without removing the gel from the glass sandwich. (Glyko, Inc.).
2. 10 mM sodium phosphate buffer, pH 7.6.
3. 10X electrophoresis electrode buffer: 1.92 *M* glycine, 0.25 *M* Tris base, pH 8.5 (Laemmli [11] buffer without SDS).
4. 10X Sodium dodecyl sulfate (SDS)/ β-mercaptoethanol (BME) [1% SDS/ 0.5 *M* BME] solution.

5. 2X de-glycosylation buffer: 40 mM sodium phosphate buffer, pH 8.6.
6. 5% Nonidet P-40 (NP-40) solution in water.
7. 2X sample loading buffer: 40% Glycerol in water.
8. Freshly made 0.15 M ANTS in 15% (v/v) acetic acid (Glyko, Inc.) (see **Note 3**).

9. Freshly made 1.0 M sodium cyanoborohydride in dimethyl sulfoxide (DMSO; see **Subheading 4.**).
10. Endoglycosidase, PNGase F (Glyko, Inc.).
11. Endoglycosidase, Endo-H (Glyko, Inc.).
12. Exoglycosidase, NANase III (Glyko, Inc.).
13. Exoglycosidase, GALase III (Glyko, Inc.).
14. Exoglycosidase, HEXase III (Glyko, Inc.).
15. Exoglycosidase, MANase II (Glyko, Inc.).
16. Exoglycosidase, MANase 6 (Glyko, Inc.).
17. ANTS-labeled oligoladder standard: polymers of glucose standards (Glyko, Inc.).
18. ANTS-labeled core standards (Glyko, Inc.).
19. Maltotetraose (G4) aliquots of 1 nmole/vial (Glyko, Inc.).
20. 5X sequence reaction buffer: 100 mM sodium phosphate, pH 5.6.
21. 100% Ethanol stored at -20° C.
22. 0.5 M phosphoric acid.
23. 0.1 M sodium hydroxide.

2.3—

Glycoproteins

1. Recombinant human factor VIII (rFVIII) from Chinese Hamster Ovary (CHO) cells (Baxter, Biotech-Hyland Division, Duarte, CA).
2. Electrophoretically purified mouse submandibular and sublingual gland mucins (USC School of Dentistry, Los Angeles, CA).

3—

Methods

The steps involved here are illustrated using the rFVIII and the mouse submandibular and sublingual gland mucins. However, any other glycoprotein may be substituted in their place for oligosaccharide analysis. This section is divided into two major divisions: one corresponding to the isolation of the individual oligosaccharide bands (**Fig. 4**), and the other relating to the sequencing of isolated bands (**Fig. 5**).

3.1—

Profile and Isolation of Individual Oligosaccharide Bands

3.1.1—

Release of the Oligosaccharides from the Glycoprotein

1. Dialyze the pure glycoprotein (in this case rFVIII or mucin), using the Slide-A-Lyzer, against 10 mM phosphate buffer, pH 7.6, for 6–10 h at 4°C, aliquot 100–200 µg of protein into 1.5 mL microtest tubes, and dry them in a centrifugal evaporating system (like the Speed Vac).
2. Dissolve one aliquot of 100–200 µg protein in 20 µL of water and add 25 µL of 2X deglycosylation buffer and 2.5 µL of SDS/BME solution. After closing the cap, boil in a water bath for 5 min, and cool to room temperature. Add 2.5 µL of NP-40 solution and mix.
3. Add 5 mU of PNGase F (2 µL) to the denatured glycoprotein and incubate at 37°C for 16 h.

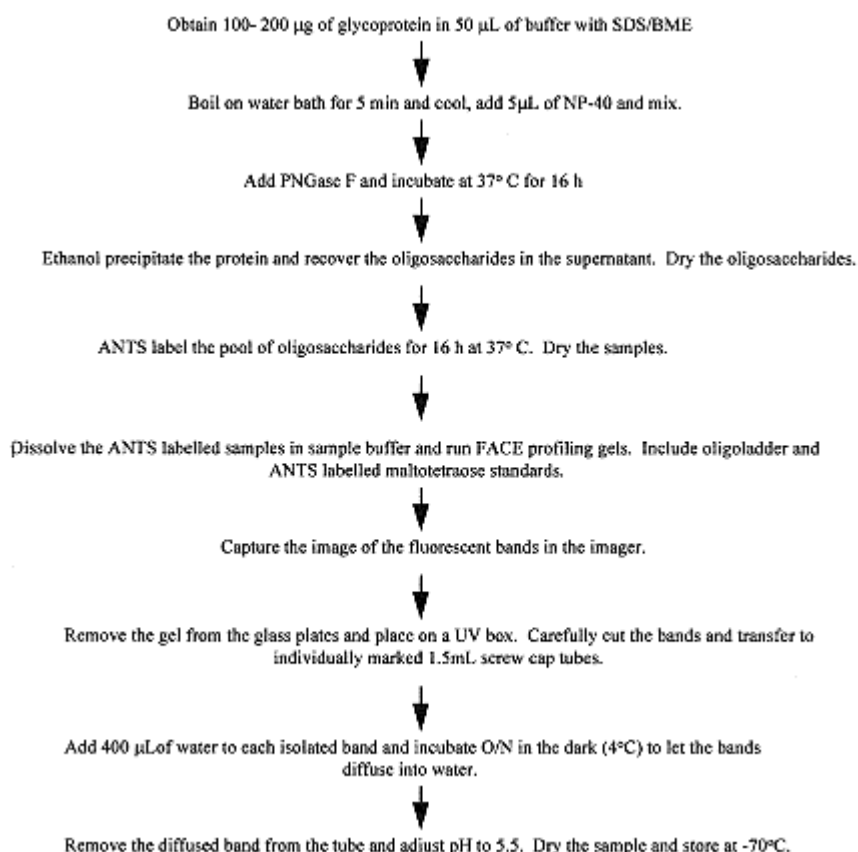


Fig. 4.

Isolation of individual ANTS-labeled oligosaccharide bands.

4. Precipitate the protein by adding 150 µL of cold 100% ethanol and keep the samples on ice for 10 min. Centrifuge the samples in a microcentrifuge for 5 min, transfer the supernatant to a fresh 1.5-mL tube, and dry the supernatant in a centrifugal evaporator.

3.1.2—

Labeling of the Oligosaccharides with ANTS

1. To the dry oligosaccharide, isolated from the glycoprotein, add 5 µL of freshly made ANTS solution followed by the addition of 5 µL of sodium cyanoborohydride, mix, briefly centrifuge, and incubate in the dark at 37° C for 16 h. Dry the samples in the centrifugal evaporator, and dissolve in 40–80 µL of IX sample loading buffer.

2. Label similarly a 1-nmol aliquot of maltotetraose (G4) with ANTS for quantitative comparison, and incubate at 37°C for 16 h. Dry the sample and make up in 80 µL of IX sample buffer.

3. Make up the prelabeled ANTS oligoladder with 100 mL of IX sample buffer.

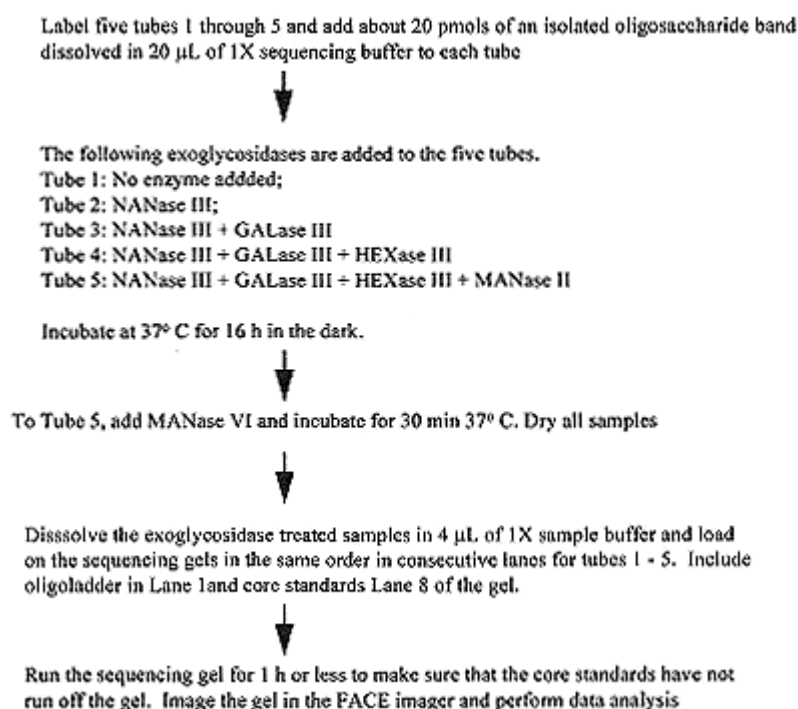


Fig. 5.
Sequence analysis of isolated ANTS-labeled oligosaccharide.

3.1.3—

Electrophoresis and Isolation of the Oligosaccharide Bands

1. Insert the gel to the cooling gel electrophoresis box, and pour cold electrophoresis buffer into the lower and upper chambers.
2. The precast polyacrylamide gel (26–30% polyacrylamide) consists of 8 lanes. After removing the combs, rinse the lanes with cold electrophoresis buffer.
3. Carefully load 4 µL of the oligoladder onto lane 1 using the flat gel-loading micropipet tips, and leave lane 2 blank to avoid crossover between the oligoglucose ladder and sample when performing analytical evaluations.
4. Load 4 µL of the ANTS-labeled oligosaccharide mixture into Lanes 3–6. Leave lane 7 blank if desired.
5. Load 4 µL of maltotetraose (G4), the equivalent of 50 pmols in Lane 8.
6. Apply 20 mA current/gel, and run for about 70–80 min or until the tracker dye reaches the bottom of the gel. This will ensure that the G4 maltotetraose does not run off the bottom of the gel (see **Note 5**).
7. Remove the gel and obtain an image of the separated bands in the FACE Imager. Label the bands as 1, 2, 3, and so on (or a similar numbering system) for identification of the bands. A profile of the rFVIII and sublingual mucin oligosaccharide bands are shown in **Fig. 6**.

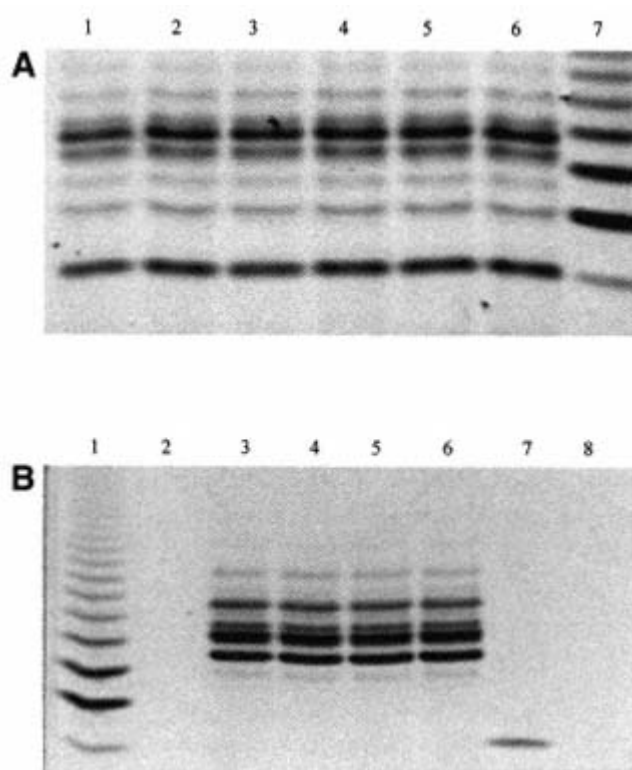


Fig. 6.

Profile of PNGase F released oligosaccharides from mouse sublingual gland mucin (**A**) and recombinant FVIII (**B**). Lane 1 in (**A**) and Lane 7 in (**B**) has the glucose ladder. Lane 7 of (**B**) has the maltotetraose (G4) quantitative standard. Lanes 1–6 (**A**) show the mucin *N*-linked oligosaccharides and lanes 3–6 in (**B**) show the rFVIII *N*-linked oligosaccharides, respectively.

8. Carefully cut the tape at one side of the gel assembly to open up the glass sandwich, and transfer the gel onto the UV light box. Make sure goggles and face shield are used in this procedure to protect from UV radiation.

9. Using a single-edge razor blade, slice the individual fluorescent bands from the gel. Transfer the gel slices to corresponding numbered tubes, add 400 μL of water, and incubate at 4° C in the dark for 16 h.

10. Remove the supernatants that contain each eluted oligosaccharide band, and add to new tubes that have been given the corresponding number. Adjust supernatants to pH 5.5 using 0.5 *M* phosphoric acid. If the pH falls below 5.5, it may be readjusted using 0.1 *M* sodium hydroxide. Dry the samples in the centrifugal evaporator. The isolated bands may be stored at -70°C for several weeks.

11. Quantitate the amount of each band isolated against maltotetraose (G4) by running a small aliquot of the isolated band. For example, the isolated band may be taken up

in 30 μL of water, and 2 μL may be loaded on a gel (after mixing with 2 μL of 2X sample loading buffer) for quantitation and storing the remainder at -70°C .

3.2—

Sequencing and Data Analysis

3.2.1—

FACE Sequencing Gels.

1. Transfer about 20 pmol of the isolated oligosaccharide (in 5 μL or less) into each of five 1.5-mL tubes (marked 1–5). Add 4 μL of 5X sequence reaction buffer to each tube. The total volume should now be 10 μL or less.

2. Set up a matrix as shown below for the addition of the sequencing exoglycosidases. The total volume in each tube is 20 μL . Tube 1 is a control with no exoglycosidases. Incubate the tubes 37°C in the dark for 16 h.

Tube #	12	3	4	5	
Oligosaccharide (~ 20 pmol)	5 μL	5 μL	5 μL	5 μL	5 μL
5X sequencing buffer	4 μL	4 μL	4 μL	4 μL	4 μL
Water (to make up volume to 20 μL)	11 μL	9 μL	7 μL	5 μL	3 μL
NANase III	—	2 μL	2 μL	2 μL	2 μL
GALase III	—	—	2 μL	2 μL	2 μL
HEXase III	—	—	—	2 μL	2 μL
MANase II	—	—	—	—	2 μL

The total volume in each tube is 20 μL . Tube 1 is a control with no exoglycosidases. Incubate the tubes at 37°C in the dark for 16 h.

3. Following the 16-h incubation, to tube # 5 only, which contains MANase II in addition to other enzymes, add 2 μL of MANase VI. Incubate for another 30 min at 37°C .

4. Dry all the five tubes in a centrifugal evaporator, and make up the volume of each tube with 4 μL of 1X sample loading buffer. The samples are now ready for loading on the sequencing gels.

5. Make up the core standards in 1X sample loading buffer to obtain a concentration of about 20 pmol/4 μL .

6. Place a pre-formed sequencing gel in the electrophoresis box, and add cold electrophoresis buffer. Prepare the sequencing gel by removing the comb and rinsing the wells with the cold buffer.

7. Load the samples in the five tubes (from **step 4**) in the middle lanes of the gel. It is important that the samples are loaded in the same order in consecutive lanes. For example, load:

Tube 1 in lane 3.

Tube 2 in lane 4.

Tube 3 in lane 5.

Tube 4 in lane 6.

Tube 5 in lane 7.

8. Load the oligoladder in lane 1 and the core standards in lane 8. Load lane 2 with the mixture of the oligosaccharides (**Subheading 3.1.2., step 3**) for comparison, or it may be left blank.

9. Carryout the electrophoresis at 20 mA/ gel for about 1 h. Note that the time of electrophoresis is shorter than that used for the isolation of the bands (**Subheading 3.1.3., step 6**).

10. Remove the gel, and capture the image of the bands using the FACE imager.

3.2.2—

Data Analysis

1. Find the band position in the gel using the FACE image analysis software. The FACE software also provides tools to specify the migration distance for G4 (maltotetraose) and G12 (polymer of 12 glucose units). Specifying these parameters, the software calibrates the migration distance for all bands in the image in terms of DP units.

2. Note the DP values for the bands in Lanes 3–7. In **Fig. 7**, the DP values for each of the bands is as follows.

Lane #	Band DP
Lane 3	6.3
Lane 4	8.4
Lane 5	6.3
Lane 6	4.8
Lane 7	3.3

3. Change in DP (Δ DP) values are interpreted as follows.

Change Form	Treatment	Δ DP	Monosaccharide lost
Lane 3–4	NANase III	2.1	NeuAc
Lane 4–5	GALase III	2.1	Gal
Lane 5–6	HEXase III	1.5	GlcNAc
Lane 6–7	MANase II	1.5	Man

(+MANase VI)

4. The number of monosaccharide residues lost by treatment of each exoglycosidase is determined by dividing the Δ DP by the average shift in Δ DP/monosaccharide (**Table 1**). Thus, for NANase III, Δ DP = 2.1 when divided by 1 gives rise to 2 NeuAc residues. Similarly, for MANase II a change in Δ DP of 1.5 divided by 0.75 (for Man) gives rise to 2 Man residues. The sequence gel for one of the rFVIII bands is illustrated in **Fig. 7** along with the change in DP values for each monosaccharide residue.
5. The band position in lane 7 (**Fig. 7**) in relation to the core standards in lane 8 determines whether the core structure is fucosylated or not. In **Fig. 7A**, the band in lane 7 aligns with the upper band in lane 8, showing that the core in this oligosaccharide is fucosylated. The lower band in lane 8 is the nonfucosylated core standard.
6. Assembling all this information starting from the core end (lane 7 and assembling towards lane 3), one can construct the structure of the oligosaccharide to be a bisialylated biantennary core fucosylated oligosaccharide (**Fig. 7B**).

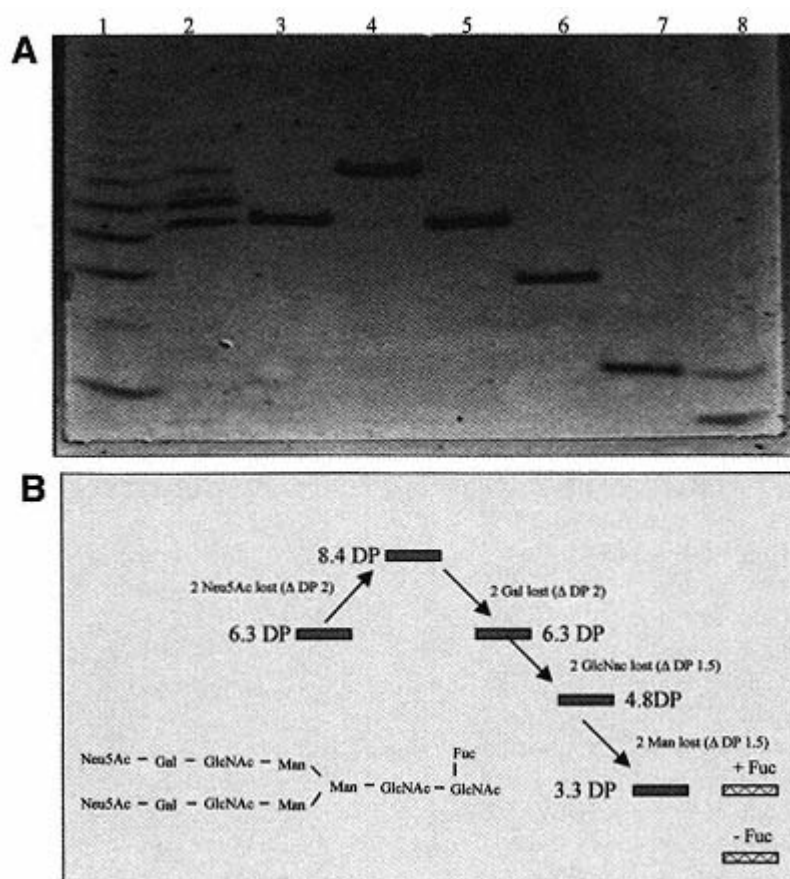


Fig. 7.

(A) Sequencing gel showing a bi-antennary, bi-sialylated core fucosylated structure isolated from the profile of rFVIII. The Δ DP shifts owing to treatment of the oligosaccharide in lane 3 with various exoglycosidases are shown in (B).

(See Subheading 3.2.2. under **Methods** for details).

4—

Notes

1. The release of *N*-linked oligosaccharides is best accomplished by enzymatic means. Other methods (like with hydrazine) have been used to release both *N*-linked and *O*-linked oligosaccharides. However, we have found the enzymatic method to be most straightforward.
2. During ethanol precipitation of the glycoprotein, following treatment with the endoglycosidases (PNGase F), it important to note that the oligosaccharides are present in the supernatants and not in the pellet.
3. It is important to keep the ANT-reagents and ANT-labeled oligosaccharides away from exposure to light as much as possible. One way to accomplish this is

to keep them in a drawer below the workbench, and take them out only during addition of reagents or other needed manipulations. Store at -70°C for longterm storage.

4. The electrophoretic gel box should be connected to a circulating bath and well cooled to 4°C. During electrophoresis, a large amount of heat is generated, and it is necessary to disperse the heat to keep the gel bands from “smiling” and deforming in shape
5. Electrophoresis may be continued after imaging the gel to improve separation of the bands. This can easily be done by returning the gel to the gel box, pouring more buffer into the upper chamber, and continuing with electrophoresis. Usually an additional 5–15 min of electrophoresis will be sufficient.
6. Frequently, the isolated bands resolve into two or more bands following treatment with NANase III. These may be reisolated from the gel at this stage as asialc-oligosaccharides before continuing with the subsequent steps in the sequencing protocol.
7. ANTS label should be made up fresh whenever possible. However, solutions made up in 15% acetic acid may be stored at -70°C for up to 2 wk. The same storage conditions apply to solutions of 1 M sodium cyanoborohydride in DMSO.
8. The removal of *N*-acetylneuraminic acid residue results in the band shifting upward in the sequencing gels as shown in **Fig. 7**. The band in lane 3 after treatment with NANase III moves up in lane 4 (a shift from DP 6.3 to DP 8.4). This is owing to loss of a negative charge associated with *N*-acetylneuraminic acid. The shift in DP value is not exactly 1/*N*-acetylneuraminic acid residue, but may vary depending on the type of the oligosaccharide being sequenced.

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19—

Application of Sucrose Synthase in the Synthesis of Nucleotide Sugars and Saccharides

Astrid Zervosen and Lothar Elling

1—

Introduction

1.1—

Overview

The realization that the oligosaccharide moieties of glycoconjugates, such as glycoproteins and glycolipids, are involved in important intra- and intercellular communication events has led to an increasing demand for the synthesis of important oligosaccharide structures as tools in analytical and therapeutic studies (see **refs. 1** and **2** for reviews). A number of efficient chemical procedures have been developed for the synthesis of the “glyco-” part (see **refs. 3–7** for review). However, for the synthesis of a given saccharide structure, an individual strategy has to be set up comprising many laborious protection and deprotection steps for stereo-controlled synthesis, which finally results in only moderate overall yields. As an example, the synthesis of *N*-acetylglucosamine (GlcNAc) involves 12 steps and needs 3 months of lab work (**7**). A scale-up of the chemical procedure often encounters environmental problems because all steps are carried out in organic solvents.

In contrast, the enzymatic synthesis of oligosaccharide structures gives high product yields in a relatively short time by stereo- and regioselective one-step reactions. All enzymatic reactions are easy to scale up and are carried out in aqueous solutions. A whole set of enzymes is now available to build up monosaccharides, phosphorylated sugars, or nucleotide sugars as well as di- and oligosaccharides (**8**). In the latter case, glycosidases (see **ref. 9** for review) and Leloir glycosyltransferases have been widely used. In many cases, Leloir glycosyltransferases are preferred because of higher product yields and an absolute stereo- and regioselectivity (**8,10**).

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Table 1
Isolation of SuSy from 18 kg Rice

Step	Activity, U	Protein, mg	Factor, fold	Yield, %
Homogenate	12,041	86,007	1.0	100.0
Filtrate	8730	24423	2.6	72.5
Sepharose Q pool	3969	1519	18.6	33.0
Ultrafiltration	2894	1131	18.3	24.1
Gel-filtration pool	1968	1108	127	16.3

Leloir glycosyltransferases catalyze the transfer of the glycosyl moiety from a nucleoside mono- or diphosphate-activated monosaccharide (nucleotide sugars) to an acceptor substrate, which is a mono or oligosaccharide or an aglycon, such as a protein, peptide, or a lipid. For large-scale synthesis of oligosaccharides, the nucleotide sugars are very expensive (e.g., UDP-Gal approx \$6000/g). Therefore, pyrophosphorylases (EC 2.7.7) have been effectively used for (1) the large-scale enzymatic synthesis of nucleotide sugars and for (2) the *in situ* regeneration of nucleotide sugars (see ref. **11** for review).

In this context, we have established a new aspect by synthesizing nucleotide sugars from the renewable resource sucrose with sucrose synthase (UDPG: D-fructose 2 glucosyltransferase, EC 2.4.1.13, SuSy). SuSy represents a unique plant glycosyltransferase, since it catalyzes the reversible cleavage of sucrose with nucleoside diphosphates to generate activated glucose and D-fructose. We have isolated SuSy from rice grains (**12,13**). The enzyme isolation was scaled up to 2000 U SuSy from 18 kg rice grains with a yield of 16% (**Table 1**). Recently, we have expressed the SuSy gene from potato in yeast (**14**). In general, both enzymes accept UDP, dUDP, dTDP, ADP, CDP, and GDP in the cleavage reaction of sucrose (**15,16**). In combination with kinases ADP-Glc, CDP-Glc and UDP-Glc were synthesized from the cheaper nucleoside monophosphates (**17**); with an additional combination of dTDP-glucose 4,6 dehydratase, dUDP- or dTDP-6-deoxy-D-xyl-4-hexulose was prepared (**17,18**).

1.2—

Enzymatic Synthesis of ADP-Glucose and N-Acetylactosamine (LacNAc)

We present here a method for the large-scale synthesis of a nucleotide sugar—the synthesis of ADP-glucose (ADP-Glc) from AMP, ATP, and sucrose (scale: 2.2 g isolated sugar) (**Fig. 1**). In contrast to our previously described method (**17**), we avoid the *in situ* regeneration of ATP with the phosphoenolpyruvate/pyruvate kinase system because of the low price for ATP and difficulties in separating ADP-Glc from pyruvate.

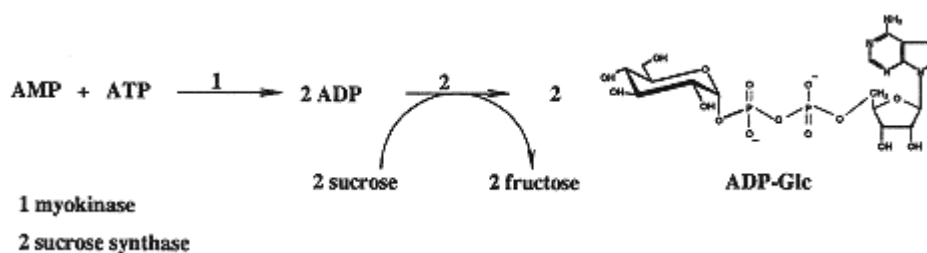
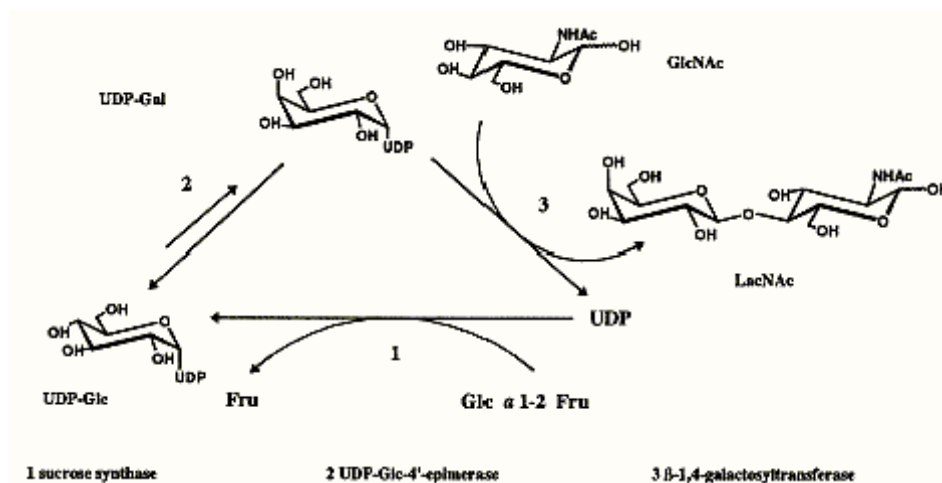


Fig. 2.

Enzymatic synthesis of LacNAc with *in situ* regeneration of UDP-Gal (22).

Fig. 2. Enzymatic synthesis of LacNAc with *in situ* regeneration of UDP-Gal (22)

ADP-Glc is the major glycosyl donor in starch biosynthesis in plants (19,20) and in the glycogen biosynthesis in bacteria (21). Starch is important as a component of, e.g., human diet and biodegradables (20). The enzymatic synthesis of ADP-Glc as outlined in Fig. 1 using myokinase and SuSy represents the first approach to producing this nucleotide sugar in gram quantities as well as to supply it for studies of starch synthase or glycogen synthase.

In the second example, we present the synthesis of the disaccharide *N*-acetylglucosamine (Galβ1, 4GlcNAc, LacNAc) with *in situ* regeneration of the nucleotide sugar UDP-Gal using SuSy and UDP-Glc 4'-epimerase (scale: 350 mg isolated LacNAc) (Fig. 2) (22). LacNAc represents an important disaccharide building block as part of terminal oligosaccharide structures, such as the selectin ligand SialylLewis^x, in complex N-glycans and O-glycans of glycoproteins (23,24).

In order to increase the enzyme productivities (g product/U of enzyme) and to reduce costs for enzymes, we used in both examples the repetitive-batch technique (Fig. 3). Here the enzymes are repetitively used after recovery by

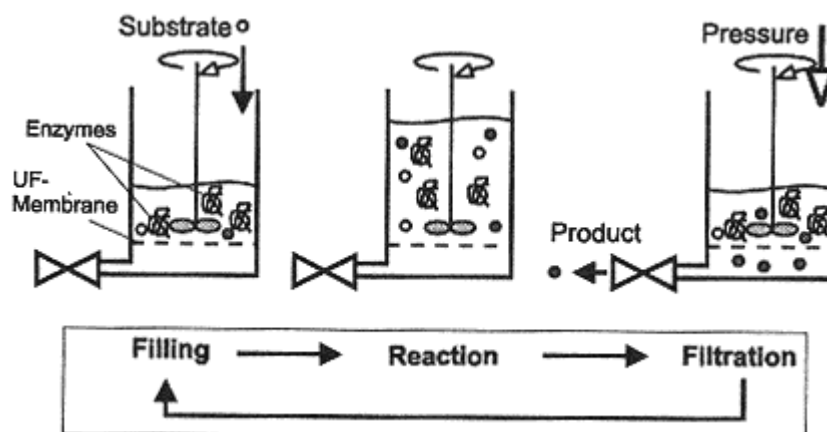


Fig. 3.

Repetitive-batch technique for enzymatic synthesis.

ultrafiltration and addition of fresh substrate solution. The excellent stability of all enzymes in the presence of sucrose, BSA (1 mg/mL) and DTT enabled us to use the enzymes over several days. However, UDP-Glc-4'-epimerase is inactivated by a so-called suicide mechanism when high concentrations of the substrates UDP-Glc and UDP-Gal or the presence of UMP and monosaccharides (e.g., Glc) forces the enzyme-bound cofactor NAD⁺ to stay in the reduced state (reductive inactivation of the enzyme) (25). A solution to this problem is the addition of fresh enzyme or the reactivation of UDP-glucose 4'-epimerase during the synthesis by the addition of the transition-state analogs dUDP- or dTDP-6-deoxy-D-xylo-4-hexulose to the reaction mixture, which we have synthesized in 0.1 to 1.0-g scale (17,18). The latter accounts also for an increase in enzyme productivity, because the enzyme is kept fully active.

With the described methods (see **Subheadings 3.2.** and **3.3.**), we have synthesized 2.8 g ADP-Glc (55.6% average yield in 10 batches) and 600 mg LacNAc (57.4% average yield in 11 batches) (**Tables 2** and **3**). In the ADP-Glc synthesis, 0.028 g/U SuSy is produced; with respect to the most expensive enzymes in the LacNAc synthesis, 0.475 g/U β 1,4GalT and 0.119 g/U UDP-Glc 4'-epimerase are synthesized.

1.3—

Isolation of ADP-Glucose (ADP-Glc) and (LacNAc)

The product isolation of ADP-Glc is performed in three steps:

1. Enzymatic cleavage of nucleotides with alkaline phosphatase from calf intestine (EC 3.1.3.1).
2. Anion-exchange chromatography.
3. Desalting (see also **Subheading 3.2.**).

Table 2
Enzymatic Synthesis of ADP-Glc by Repetitive-Batch Technique (10 Batches)

A. Details for Each Batch

Batch no.	Amount of ADP-Glc, mmol	ADP-Glc, mg	Yield ^a , %
1	0.4	254	50.0
2	0.4	251	50.0
3	0.47	296	58.8
4	0.47	298	58.8
5	0.39	240	48.8
6 ^b	0.48	303	60.0
7	0.41	255	51.3
8	0.48	304	60.0
9	0.47	300	58.8
10	0.48	303	60.0
Average/sum	4.45	2804	55.6

B. Enzymes, Yield, Enzyme Productivities, and Time of Synthesis

Sucrose synthase (SuSy) (U)	100
Myokinase (MK) (U)	20
Average yield (%)	55.6
Enzyme productivity (g ADP-Glc/U SuSy)	0.028
Enzyme productivity (g ADP-Glc/U MK)	0.14
Time (d)	10

^aWith reference to the amount of ATP and AMP (0.8 mmol) at the beginning of the respective batch.

^bAddition of 10 U myokinase.

We recommend this isolation method for all nucleotide sugars produced by SuSy. In order to avoid the separation of nucleotides and nucleotide sugar by different anion-exchange chromatography columns (26), we first cleave all nucleotides with alkaline phosphatase. Alkaline phosphatase is separated from the product solution by ultrafiltration. Subsequently, ADP-Glc is isolated from the raw product solution by anion-exchange chromatography using Sepharose Q FF. Here care must be taken of the pH value during the column run. The raw product solution is loaded at a pH of 6.5. Since all subsequent elution steps are performed without any buffer salts (distilled water), the partial cleavage of ADP-Glc would rapidly cause an alkaline pH during elution. Since ADP-Glc is not stable at alkaline pH > 8.0 (27) as well as to avoid an alkaline pH on the column caused by decomposition of ADP-Glc, we elute the product at pH 2.0.

Table 3
Enzymatic Synthesis of LacNAc with *In Situ* Regeneration of UDP-Gal (22)

Batches/repetitions/volume (mL)	1/10/25
β 1,4 Galactosyltransferase (GalT) (U)	1.25
UDP-Glc 4'-epimerase (epimerase) (U)	5
SuSy (U) 10 Average Yield (%)	57.4
Space-time yield (g product/L batch in 1 d)	2.2
Cycle number (mol product/mol UDP-Glc)	5.7
Amount of synthesized LacNAc (g)	0.594
Enzyme productivity (g LacNAc/U GalT)	0.475
Enzyme productivity (g LacNAc/U epimerase)	0.119
Enzyme productivity (g LacNAc/U SuSy)	0.059

Table 4
Isolation of ADP-Glc

Step	Yield, %	Amount ADP-Glc, mg
ADP-Glc from enzymatic synthesis	100	2804
Cleavage of nucleotides with alkaline phosphatase	94.1	2639
Ultrafiltration	92.7	2599
Anion-exchange column	89.8	2517
Salt precipitation	86.6	2429
Gel filtration	80.4	2254
Lyophilization	78.5	2201

For elution, we use NaCl instead of LiCl because of the excellent stability of the Na salt of ADP-Glc. The last step of the product isolation is desalting of ADP-Glc by gel filtration. In order to reduce the amount of salt loaded onto the column, a part of NaCl is precipitated with ethanol. With the described method, we have purified 2.2 g ADP-Glc (**Table 4**). The purity of the product was 76% (w/w) as determined by HPLC containing NaCl and water. The overall yield was 43.6%.

The product isolation of LacNAc is accomplished in four steps:

1. Enzymatically cleaving sucrose with invertase.
2. Separation of LacNAc from large quantities of monosaccharides by ligandexchange chromatography with a cation exchanger in Ca^{2+} -form.
3. Anion-exchange chromatography.
4. Gel filtration.

Table 5
Isolation of LacNAc

Step	Yield, %	Amount LacNAc, mg
LacNAc from enzymatic synthesis	100	594
Cleavage of sucrose with invertase	86	511
Cation-exchange (AG50W-X8) column	83.2	494
Anion-exchange column (Dowex 1 × 2, Cl ⁻ -form)	81.3	483
Gel-filtration Biogel P2 column	76.3	453
Anion-exchange column (Dowex 1 × 2, Cl ⁻ -form)	73.2	435
Gel-filtration Biogel P2 column	67.7	402
Cation-exchange (AG50W-X8) column	59.9	356

In order to facilitate the separation of the disaccharides sucrose and LacNAc, sucrose is cleaved to yield glucose and fructose with invertase from yeast (EC 3.2.1.26), which is subsequently separated from the product solution by ultrafiltration. In the second step, LacNAc is separated from the monosaccharides fructose, glucose, and GlcNAc by ligand-exchange chromatography, because they form Ca²⁺-complexes with different stabilities. LacNAc is further separated from nucleotides, nucleotide sugars, and buffer salt (HEPES) by anion-exchange chromatography. In order to bind the buffer salt (HEPES) on the anion exchanger, the pH has to be adjusted to 8.5. In the subsequent gelfiltration step, LacNAc is desalted and separated from residual Glc and GlcNAc. In order to improve the purity of the final product, we have repeated the anion-exchange, gel-filtration, and cation-exchange step. In this way, we have purified 356 mg LacNAc (**Table 5**). The purity of the product was 77% (w/w) as determined by HPLC containing 4.2% (w/w) GlcNAc and 18.7% (w/w) NaCl. The overall yield of the LacNAc synthesis was 34.4%.

2—

Materials.

2.1—

Isolation of Sucrose Synthase from Rice

2.1.1—

Assay of Sucrose Synthase

1. Buffer A: 200 mM HEPES-NaOH, pH 7.2.
2. 2 M Sucrose in buffer A.
3. 20 mM UDP in buffer A (make fresh as required).

2.1.2—

Analytical HPLC Method

1. HPLC system with high-precision pump (Modell 300), an automated sampler (Modell GINA 160) and UV spectrophotometer (Modell 160) from GynkoteK (München, Germany).

2. HPLC column (Hypersil ODS column, 5- μ m particle size, 250 $\times$ 4.6 mm) from Machery Nagel (Düren, Germany).
3. Centrifuge: Biofuge A from Heraeus Christ (Osterode, Germany).
4. Solvent for ion-pair, reverse-phase HPLC: 100 mM potassium acetate, pH 5.6, containing 0.013% (v/v) *n*-octylamine and 5% (v/v) methanol (methanol highpurity reagent from Fisons, Loughborough, Great Britain). Flow rate: 1 mL/min.
5. UMP (Na salt), UDP-glucose (Na salt), UDP (Na salt) from Sigma (Deisenhofen, Germany).

2.1.3—

Photometric Assay for the Determination of the UDP-Glucose Concentration

1. UV-160 spectrometer with a CPS temperature controller and a CPS cell positioner from Shimadzu (Düsseldorf, Germany).
2. Buffer B: 50 mM Tris-HCl, pH 8.7.
3. 30 mM NAD in buffer B (make fresh as required).
4. 1.1 U/mL UDP-glucose dehydrogenase in buffer B from Sigma (make fresh as required).

2.1.4—

Protein Assay

1. Bradford reagent: Solve 100 mg Coomassie brilliant blue G-250 from Fluka (Basel, Switzerland) in 50 mL 95% ethanol. Add 100 mL 85% (w/v) phosphoric acid and fill up to 1 L solution with distilled water. After 24 h, filter the solution over a plaited filter.
2. Bovine serum albumin (BSA).

2.1.5—

Isolation of Sucrose Synthase from Rice

2.1.5.1—

Preparation of Crude Extract

1. 9 kg Common unpolished rice from a food wholesaler (Rila, Stemwede, Germany).
2. Buffer C: 50 mM HEPES-NaOH, pH 7.2 (18 L).
3. Commercial Waring Blender from Waring (New Hartford, CT).

2.1.5.2—

Pressure Filtration

1. Pressure filter Merkur (type EF 45/65 CW) from Seitz Enzinger Noll (Mannheim, Germany) equipped with a Seitz Supra 100 filter (45 cm diameter).
2. 5% Polyethylene glycol (PEG) 4000 in distilled water (1.2 L).

2.1.5.3—

Anion-Exchange Chromatography.

1. 1.4 L Sepharose Q-FF from Pharmacia (Freiburg, Germany) filled in a column (11.3 cm inner diameter).
2. Buffer D: 50 mM HEPES-NaOH pH 8.0 (5 L).
3. Buffer E: buffer D with 0.1 M KCl (23 L).
4. Buffer F: buffer D with 0.4 M KCl (7.5 L).

5. A pump from Ismatec (Type MV-Z) (Wertheim-Mondfeld, Germany), a singlepath monitor UV-1 with a S-2 UV flowthrough cell (280 nm), and a recorder from Pharmacia.
6. 0.1 M Potassium acetate, pH 4.0, with 1 M NaCl (5 L).
7. 2 M NaOH (2 L).
8. 50 mM HEPES-NaOH, pH 7.2, with 1 M NaCl (5 L).

2.1.5.4—

Ultrafiltration

1. A spiral cartridge concentrator with a S1Y30 cartridge (30-kDa cutoff) from Amicon (Witten, Germany) connected to a pump from Ismatec (Type MV-Z).
2. A stirred cell (Type 8050) from Amicon (Witten, Germany) with a YM30 membrane (30-kDa cutoff).

2.1.5.5—

Gel Filtration

1. 1.57 L Superdex 200 prep grade from Pharmacia filled in a XK 50/80 column (inner diameter: 5 cm) connected to an FPLC equipment with a HighLoad pump P50 from Pharmacia.
2. Buffer G: 200 mM HEPES-NaOH, pH 7.2, with 150 mM KCl.

2.2—

Enzymatic Synthesis and Isolation of ADP-Glc

2.2.1—

Analytical HPLC Method for Nucleosides, Nucleotides, and ADP-Glc

1. HPLC system, *see Subheading 2.1.2.*
2. HPLC column (Hypersil ODS column, 5- μ m particle size, 250 $\times$ 4.6 mm) from Machery Nagel.
3. Centrifuge: Biofuge A from Heraeus Christ (Osterode, Germany), Sigma 3 from Sigma Laborzentrifugen GmbH (Osterode am Harz, Germany).
4. Solvent for ion-pair, reverse-phase HPLC: 100 mM potassium acetate, pH 4.6, containing 0.013% (v/v) *n*-octylamine and 8% (v/v) methanol (methanol high-purity reagent from Fisons, Loughborough, Great Britain). Flow rate 0.8 mL/min.
5. Adenosine, AMP (Na salt), ADP-Glc (Na salt), ADP (Na salt) from Sigma.

2.2.2—

Synthesis of ADP-Glc

1. Buffer H: 200 mM HEPES-NaOH, pH 7.5, containing 500 mM sucrose, 3 mM 1,4 dithiothreitol (DTT), and 0.125 mM MgCl₂ (make fresh as required).
2. 40 mM AMP (Na salt) in buffer H (make fresh as required).
3. 40 mM ATP (Na salt) in buffer H (make fresh as required).

4. 10 U/mL partially purified SuSy from rice grains (see **Subheading 3.1.**) or recombinant sucrose synthase (SuSy) from potato in buffer H (make fresh as required). Sucrose synthase was purified as previously described (**13**) and is stable at -20°C in 200 mM HEPES-NaOH, pH 7.6, for up to 6 mo. (**Attention:** Sucrose synthase [S 6379] available from Sigma cannot be used because of contaminating nucleotide phosphatases).

5. 1 U/mL myokinase from rabbit muscle (EC 2.7.4.3) from Sigma in buffer H (make fresh as required).
6. 10 mg/mL BSA in buffer H (make fresh as required).
7. 5 % (w/w) PEG 4000 solution.
8. Stirred ultrafiltration cell (Amicon, model 8050, equipped with a membrane YM 10, cut off 10 kDa, Amicon GmbH).

2.2.3—

Isolation of ADP-Glc

1. Alkaline phosphatase from calf intestine (EC 3.1.3.1) (Boehringer Mannheim, Mannheim, Germany).
2. Stirred ultrafiltration cell (Amicon, model 8050, equipped with a membrane YM 10, cut off 10 kDa, Amicon GmbH).
3. 460 mL Sepharose Q Fast Flow (Pharmacia) packed in a column (A 50, 30 cm, Pharmacia) and stored in 20% ethanol at 4°C.
4. Preparative chromatography system with fraction collector (FRAC-300), peristaltic pump P-1, monitor UV-1, conductivity monitor, recorder (REC 101) from Pharmacia (Freiburg, Germany).
5. Distilled water adjusted to pH 2.0 with HCl.
6. 0.4 M NaCl adjusted to pH 2.0 with HCl.
7. 1 M NaCl.
8. Rotary evaporator with vacuum pump.
9. Ethanol (w/w 99.6%).
10. 500 mL Sephadex G 10 (Pharmacia) packed in a column (A 26, 100 cm, Pharmacia) and stored in 0.1% NaN₃ at 4°C.
11. Cold-storage chamber at 4°C.
12. Freeze-dryer (model Lyovac GT3, Leybold AG, Hanau, Germany).

2.3—

Enzymatic Synthesis and Isolation of (LacNAc)

2.3.1—

Analytical Methods

2.3.1.1—

HPLC.

1. HPLC system (see **Subheading 2.1.2.**) with additional column heater.
2. Two Aminex HPX-87H columns (300 × 7.8 mm each, Bio-Rad, München, Germany).
3. Solvent: 4 mM H₂SO₄.

4. LacNAc and GlcNAc from Sigma.

2.3.1.2—

Thin-Layer Chromatography (TLC)

1. TLC plates: aluminum-backed, silica gel 60, fluorescent indicator F₂₅₄, layer thickness 0.2 mm (20 × 20 cm).
2. TLC solvent system: acetonitrile : 0.1 M NH₄Cl 75:25 (v/v).
3. Spray solution: 20 mg naphthoresorcinol, 40 mg diphenylamine, 10 mL ethanol, and 0.4 mL sulfuric acid.

2.3.2—

Synthesis of LacNAc

1. Buffer I: 200 mM HEPES-NaOH, pH 7.2, 500 mM sucrose, 1 mM 1,4 DTT, 25 mM KCl, 1 mM MnCl₂.

2. 10 mM UDP-glucose (Na salt) in buffer I (make fresh as required).
3. 100 mM GlcNAc in buffer I (make fresh as required).
4. 1 mM dUDP or dTDP-6-deoxy-D-xyl/o-4-hexulose in buffer I (make fresh as required).
5. 10 mg/mL bovine serum albumin in buffer I (make fresh as required).
6. 0.5 U/mL β 1,4 galactosyltransferase from human milk (Boehringer Mannheim) in buffer I (make fresh as required).
7. 2 U/mL UDP-Glc 4'-epimerase from *Saccharomyces cerevisiae* (Sigma) in buffer I (make fresh as required).
8. 4 U/mL sucrose synthase from rice grains in buffer I (make fresh as required).
9. 5 % (w/w) PEG 4000 solution.
10. Stirred ultrafiltration cell (Amicon, model 8050, equipped with a membrane YM 10, cutoff 10 kDa, Amicon GmbH).

2.3.3—

Product Isolation

1. 25,000 U/mL invertase from *S. cerevisiae* (Sigma) in buffer K (200 mM HEPES-NaOH, pH 7.2, 500 mM sucrose, 1 mM 1,4 DTT, 0.1% BSA, 25 mM KCl).
2. Polarimeter (polarimeter 241, Perkin-Elmer, Überlingen, Germany).
3. Stirred ultrafiltration cell (see **Subheading 2.2.2.**).
4. Rotary evaporator with vacuum pump.
5. Preparative chromatography system (see **Subheading 2.1.5.**).
6. 700 mL AG50W-X8 cation-exchange resin (Ca^{2+} -form, Bio-Rad) packed in a column (XK 50, 60 cm, Pharmacia) stored at 4°C.
7. 1 M CaCl_2 .
8. 150 mL Dowex 1 $\times$ 2 anion-exchange column (100–200 mesh, Cl^- -form, Serva, Heidelberg, Germany) packed in a column (C-26, 40 cm, Pharmacia) and stored at 4°C.
9. 1 M NaCl.
10. 440 mL Biogel P2 (Bio-Rad) packed in a column (XK 26, 100 cm, Pharmacia) and stored in 0.1% NaN_3 at 4°C.
11. Freeze-dryer (see **Subheading 2.2.3.**).

3—

Method

3.1—

Isolation from Sucrose Synthase from Rice

3.1.1—**Assay of Sucrose Synthase**

The sucrose synthase activity is determined by the cleavage reaction yielding UDP-glucose and fructose from sucrose and UDP.

1. Mix 550 μL buffer A, 250 μL sucrose, 100 μL UDP, and 100 μL sucrose synthase solution (with different enzyme activities).
2. Incubate the solution 10 min at 30°C, and stop the enzyme reaction by heating the solution at 95°C for 5 min.
3. Determine the UDP-glucose concentration with the HPLC method (**Subheading 3.1.2.**) or the photometric assay (**Subheading 3.1.3.**).

4. 1 U Sucrose synthase—catalyze the synthesis of 1 μmol UDP-glucose/min under standard conditions.

3.1.2—

Analytical HPLC Method

Samples were analyzed by the described ion-pair HPLC. After stopping an aliquot from the reaction mixture by heat (see **Subheading 3.1.1., step 2**) particulate matter must be removed from the sample by centrifugation at 12,000 rpm (18000g) for 10 min with Biofuge A. All solutions used in HPLC must be filtered with a 0.22- μm filter, and degassed using a vacuum pump to avoid particulate matter and air bubbles. The UDP-glucose concentration was read from a calibration chart (0.2–3 mM) of the compound vs peak area (absorption at 254 nm).

3.1.3—

Photometric Assay for the Determination of the UDP-Glucose Concentration

UDP-glucose dehydrogenase (EC 1.1.1.22) catalyzes the oxidation of 1 mol UDP-glucose with 2 mol NAD to UDP-glucuronic acid and NADH. The formation of NADH can be determined by measuring the absorption at 340 nm.

1. The measurement of NADH takes place in the photometer at 25°C.
2. Mix 885 μL buffer B, 70 μL NAD, and 35 μL sucrose synthase assay, and start the enzymatic reaction by adding 10 μL UDP-glucose dehydrogenase.
3. Calculate the concentration of UDP-glucose from the difference of the absorption at the beginning and at the end of the enzymatic reaction.

3.1.4—

Protein Assay.

1. Mix up samples containing 10–100 μg BSA in 1 mL buffer for the calibration chart.
2. Mix 100 μL sample and 900 μL Bradford reagent, and incubate the sample for 7 min at room temperature.
3. The absorption is measured at 595 nm.

3.1.5—

Isolation of Sucrose Synthase from Rice

Sucrose synthase from rice is isolated in five steps. At the end of every step, take a sample to determine the sucrose synthase activity and the protein concentration. From 18 kg rice, 2000 U sucrose synthase (yield: 16%) could be isolated with a specific activity of 18 U/mg (**Table 1**).

3.1.5.1—

Preparation of the Crude Extract

1. Mix 9 kg rice and 9 kg buffer C, and let the grains swell overnight.
2. Strain the grains, and resuspend them in 9 kg buffer C.
3. Homogenize the rice for 1.5 min in three batches (each consisting of 3 kg rice and 3 kg buffer) in a Waring Blender.

4. Take a sample, and determine the activity of sucrose synthase after centrifugation of the solution in Biofuge A.
5. Use the homogenized batches immediately for the pressure filtration.

3.1.5.2—***Pressure Filtration***

1. Treat the Seitz Supra 100 filter with 400 mL of a 5% PEG 4000 solution.
2. Filter each homogenized batch under positive pressure in a pressure filter.
3. Hold the pressure for 5 min at 10^5 Pa (1 bar) and 1.5×10^5 Pa (1.5 bar), and increase it finally to 4×10^5 Pa (4 bar).
4. Take a sample, and determine the activity of sucrose synthase and the protein concentration.
5. Use the filtrates immediately for the anion-exchange chromatography.

3.1.5.3—***Anion-Exchange Chromatography***

1. Equilibrate the Sepharose Q FF with 10 L buffer D with a linear flow rate of 1.1 cm/min.
2. Load the filtrate of the rice homogenate with a linear flow rate of 1.1 cm/min on the column.
3. Wash the column with 15–20 L buffer E with a linear flow rate of 2.2 cm/min until the absorption at 280 nm has dropped to a constant value.
4. Elute sucrose synthase with a linear salt gradient (7.5 L buffer E and 7.5 L buffer F) with a linear flow rate of 0.77 cm/min.
5. Determine the activity of sucrose synthase in the fractions (fraction size: 100 mL) with the sucrose synthase assay followed by the photometric analysis of the UDPglucose concentration.
6. Adjust the pH of the pool of the fractions containing sucrose synthase immediately to 7.2.
7. The solution can be stored at 4°C overnight.
8. Take a sample, and determine the activity of sucrose synthase and the protein concentration.
9. Regenerate the Sepharose Q FF by washing with 5 L potassium acetate, pH 4.0, with 1 M NaCl, 10 L distilled water, 2 L 2 M NaOH, 10 L distilled water, 5 L 50 mM HEPES-NaOH, pH 7.2, with 1 M NaCl, and finally with 10 L distilled water with a linear flow rate of 2.2 cm/min.
10. Store the Sepharose Q FF in 0.1% sodium azide.

3.1.5.4—***Ultrafiltration***

1. Concentrate the solution with the spiral cartridge concentrator to 300 mL.
2. Wash the filter with 150 mL buffer C.
3. Concentrate the solution with the stirred cell to 300 mL.
4. Take a sample to determine the activity of sucrose synthase and the protein concentration.

3.1.5.5—***Gel Filtration***

1. Elute the column with buffer G with a linear flow rate of 0.5 cm/min.

2. Load 50 mL of the filtrate (see **Subheading 3.1.5.4.**) on the column.
3. Determine the activity of sucrose synthase in the fractions (fraction size: 10 mL) with the sucrose synthase assay (**Subheading 3.1.1.**) followed by the photometric determination of the UDP-glucose concentration.

4. Take a sample of the pool of the fractions containing sucrose synthase activity, and determine the enzyme activity and the protein concentration.

5. Store aliquots of this pool at -20°C.

3.2—

Enzymatic Synthesis and Isolation of ADP-Glc

3.2.1—

Analytical HPLC Method for Nucleosides, Nucleotides, and ADP-Glc

Samples are prepared as described in **Subheading 3.1.2**. The adenosine, AMP, ADP-Glc and ADP concentration was read from calibration charts (0.2–3 mM) of the compounds vs peak area (absorption at 254 nm).

3.2.2—

Synthesis of ADP-Glc.

In order to maximize enzyme productivity, the repetitive-batch technique is used (*see Fig. 3*). In one batch of a volume of 100 mL, nearly 0.44 mmol (280 mg, 55% yield with reference to the sum of AMP and ATP) is synthesized. In order to synthesize 2.8 g ADP-Glc, 10 batches have to be carried out (*see Table 2*).

1. Start the synthesis with 10-mL reaction solutions of 40 mM AMP, 40 mM ATP, 10 U/mL sucrose synthase, 1 U/mL myokinase, and 10 mg/mL BSA, respectively, which are added to 50 mL buffer H (final volume 100 mL).
2. After a sterile filtration, stir the solution gently for 4 h at 30°C.
3. Check the concentration of formed ADP-Glc by HPLC (*see Note 1*): Take out an aliquot of 20 µL from the reaction solution, add 80 µL distilled water, and heat for 5 min at 95°C (enzyme reactions are stopped). Determine the yield of ADP-Glc from the peak area of the HPLC chromatogram. When a yield of approx 55% is obtained (**Table 2**), enzymes are separated from the reaction solution by ultrafiltration.
4. In order to avoid the loss of enzymes by adsorption, soak first the ultrafiltration membrane in a 5% PEG 4000 solution for 30 min and wash it thoroughly with distilled water.
5. Concentrate the reaction solution to a volume of 10 mL. Keep the filtrate, and store it at -20°C (stable for some weeks).
6. Start the second batch by addition of 10 mL of the solutions containing 40 mM AMP, 40 mM ATP to the concentrate (*see step 5*), and add 70 mL buffer H (final volume of the second batch is also 100 mL).
7. After the sterile filtration, stir the reaction solution gently for 4 h at 30°C. Repeat **steps 3–6**.
8. Stop synthesizing ADP-Glc after batch no. 10.

3.2.3—

Isolation of ADP-Glc

3.2.3.1—

Cleavage of Nucleotides with Alkaline Phosphatase

In order to simplify the isolation of ADP-Glc, the nucleotides AMP, ADP, and ATP are enzymatically cleaved with alkaline phosphatase.

1. Start the cleavage of the nucleotides by addition of 2 U alkaline phosphatase/mL raw product solution (see **Note 2**).
2. Stir the solution gently overnight at 30°C.
3. Analyze the solution by HPLC for the presence of nucleotides (see **Subheading 3.1.2.**). After the complete cleavage of the nucleotides, separate the raw product solution from alkaline phosphatase by ultrafiltration.
4. Analyze the filtrate by HPLC, and determine the concentration of ADP-Glc (mg/ mL). Store the solution at 4°C overnight or at -20°C over 2–4 d until the next step.

3.2.3.2—

Anion-Exchange Chromatography

ADP-Glc is isolated from the raw product solution by anion-exchange chromatography. This step is scaled up to isolate 1.3 g ADP-Glc from the product solution (**Table 4**). In order to isolate 2.6 g of ADP-Glc, two runs have to be carried out.

1. Equilibrate the Sepharose Q FF with distilled water at a flow rate of 12 mL/min at room temperature.
2. Calculate (from **Subheading 3.2.2.1., steps 3–8**) the volume of raw product solution containing 1.3 g ADP-Glc. Adjust the pH of this volume of raw product solution to 6.5 and load onto the column with a flow rate of 8 mL/min.
3. Wash the column with distilled water (adjusted to pH 2.0 with HCl) at a flow rate of 12 mL/min until the conductivity and the UV-absorption (254 nm) have dropped and are stabilized.
4. Elute ADP-Glc with 0.4 M NaCl (pH 2.0 adjusted with HCl) at a flow rate of 12 mL/min and a fraction size of 18 mL.
5. Pool the fractions containing ADP-Glc (absorbance at 254 nm). Adjust the pH of the pool to 7.0.
6. Analyze the pool solution by HPLC for concentration of ADP-Glc, and store the solution overnight at 4°C for 2–4 d at -20°C until the next step.

3.2.3.3—

Desalting

The pool solution is desalted in two steps by precipitation of NaCl followed by gel filtration on Sephadex G 10.

1. Concentrate the pool solution (from **Subheading 3.2.3.2.**) in a rotary evaporator at 20–25 mbar and 30–35°C until NaCl precipitates.
2. Add distilled water until the salt is redissolved and determine the volume of the solution.
3. Precipitate NaCl by addition of 50% (v/v) ethanol.
4. Separate the precipitate by centrifugation (5 min, 3000 U/min, Sigma 3 centrifuge).

5. Concentrate the solution at 20–25 mbar and 30–35°C with a rotary evaporator until NaCl precipitates, and resolve the salt by addition of distilled water. Check the concentration of this solution by HPLC.
6. Equilibrate the column with Sephadex G10 with distilled water at 4°C at a flow rate of 1 mL/min.

7. Load a volume of the solution containing a maximum of 200 mg ADP-Glc onto the column, and elute with distilled water at a flow rate of 1 mL/min and a fraction size of 10 mL.
8. Pool the fractions containing ADP-Glc (absorption at 254 nm), and store it at -20°C.
9. Repeat **steps 7 and 8**.
10. After lyophilization in a freeze-dryer, analyze the purity of ADP-Glc by dissolving 1 mg of lyophilized product in 1 mL distilled water, and determine the concentration of ADP-Glc by HPLC. The purity should be >75%. If the purity of the product is not high enough, repeat the gel-filtration step.

3.3—

Enzymatic Synthesis and Isolation of LacNAc

3.3.1—

Analytical Methods

3.3.1.1—

HPLC

Samples are prepared as described in **Subheading 3.1.2**. The LacNAc and GlcNAc concentrations were read from the calibration charts of the compounds (0.05–5 mM) vs the peak area for GlcNAc and vs peak height for LacNAc.

3.3.1.2—

Thin-Layer Chromatograph (TLC)

LacNAc and monosaccharides were separated by TLC in samples treated with invertase. The fluorescent spots of UDP, UMP, and UDP-Glc (R_f -values: UDP 0.05, UMP 0.16, UDP-Glc 0.16) were visualized by a UV lamp (wavelength 254 nm, blue spots). For visualization of LacNAc and monosaccharides, the plates were treated with spray solution, dried, and heated (110°C) until spots of different colors appeared (R_f values: LacNAc [brown] 0.32, D-fructose [blue] 0.39, D-glucose [blue] 0.39, GlcNAc [brown] 0.58).

3.3.2—

Synthesis of LacNAc.

In order to improve the enzyme productivities, the repetitive-batch technique is used (**Fig. 3**). UDP-Glc-4'-epimerase is inactivated during synthesis in the presence of different monosaccharides and UMP or at high concentrations of UDP-Glc and UDP-Gal, respectively (**22**). The enzyme is reactivated by the addition of dNDP-6-deoxy-D-xylo-4-hexulose ($N = T, U$) to the reaction mixture (see Note **3**).

1. Make up a reaction solution by mixing 2.5 mL each of the following solutions: 10 mM UDP-Glc, 100 mM GlcNAc, 1 mM dUDP-, or dTDP-6-deoxy-D-xylo-4-hexulose (these compounds were synthesized as previously described [**17,18,22**] and stored at -20°C), 10 mg/mL BSA, 0.5 U/mL β 1,4 galactosyltransferase, 2 U/mL UDP-Glc-4'-epimerase, and 4 U/mL sucrose synthase (SuSy was purified from rice as previously described [**13**] and stored at -20°C in 200 mM HEPES-NaOH, pH 7.2, for several months). Add 7.5 mL buffer I and adjust the pH to 7.2 (final volume 25 mL).

2. After a sterile filtration, stir the solution gently overnight at 30°C for 22 h.
3. Check the concentration of formed LacNAc by HPLC: Take out an aliquot of 20 µL from the reaction solution, add 80 µL distilled water, and heat for 5 min at 95°C (enzyme reactions are stopped). Determine the yield of LacNAc from the peak height of the HPLC chromatogram. When a yield of approx 100% is obtained (**Table 5**), the enzymes are separated from the reaction solution by ultrafiltration.
4. In order to avoid the loss of enzymes by adsorption, soak first the ultrafiltration membrane (YM 10) in a 5% PEG 4000 solution for 30 min, and wash it thoroughly with distilled water.
5. Dilute first the reaction solution to a total volume of 50 mL buffer I. Concentrate the reaction solution to a volume of 25 mL. Repeat the latter steps three times, and adjust the volume to 17.5 mL in the last concentration step. Combine all filtrates, and store at -20°C (stable for up to 1 mo).
6. Start the second batch by addition of 2.5 mL each of the following solutions: 10 mM UDP-Glc, 100 mM GlcNAc, and 1 mM dUDP or dTDP-6-deoxy-D-xyl-4-hexulose (final volume 25 mL, see **Note 3**).
7. After a sterile filtration, stir the solution gently overnight at 30°C.
8. Check the yield for LacNAc formation by HPLC after 24 h. When a yield of 85–90% is found, the second batch is treated as described in **steps 5** and **6**. In this way, approx 600 mg LacNAc are synthesized in 11 batches over 11 d (**Table 3**). Because of the loss of enzyme activity by sterile filtration, ultrafiltration, and inactivation during the synthesis, the yield will decrease to approx 40–45%, whereas the incubation time will increase to approx 30 h (see **Note 4**).

3.3.3—

Isolation of LacNAc

The isolation starts with a raw product solution containing 120 mg of the synthesized LacNAc. In order to isolate 600 mg LacNAc, the procedure must be repeated five times (**Table 5**).

3.3.3.1—

Enzymatic Cleavage of Sucrose

1. Preincubate 25000 U/mL invertase in buffer K for 2 h at 45°C.
2. The reaction is started by addition of 10 µL invertase solution/mL raw product solution.
3. Stir the solution gently for 2 h at 45°C.
4. Follow the cleavage of sucrose by a polarimeter. After 2 h, sucrose is completely cleaved and a constant value is indicated by the polarimeter.
5. Analyze the raw product solution by HPLC for LacNAc concentration (the solution can then be stored overnight at 4°C or over several days at -20°C).
6. The solution is filtered through a ultrafiltration membrane in a ultrafiltration cell equipped with a membrane YM10 to separate the invertase.
7. Analyze the filtrate by HPLC for LacNAc concentration.
8. Store the filtrate overnight at 4°C or over several days at -20°C until the next step.

3.3.3.2—***Ligand Exchange Chromatography on AG50W-X8***

1. Concentrate the solution in a rotary evaporator at 20–25 mbar and 30–35°C to a volume of 30 mL.
2. Equilibrate the cation-exchange (AG50W-X8) column with 1 M CaCl₂ at a flow rate of 5–6 mL/min at room temperature until the conductivity at the outlet of the column is equal to the inlet of the column.
3. Wash the column with distilled water at a flow rate of 5–6 mL/min until the conductivity drops to zero.
4. During the separation of the saccharides, the column is run at a flow rate of 0.5 mL/min with a fraction size of 6.5 mL.
5. LacNAc elutes before Glc, followed by GlcNAc and fructose. Thus, LacNAc can be isolated from fructose and large quantities of Glc and GlcNAc.
6. Analyze the fractions by TLC.
7. Pool the fractions containing LacNAc, and adjust the pH to 7.0.
8. Analyze the pool by HPLC for LacNAc concentration.
9. Store the solution overnight at 4°C or over several days at -20°C until the next step.

3.3.3.3—***Anion Exchange***

1. Equilibrate the anion-exchange column (Dowex 1 × 2, Cl⁻-form) with distilled water at a flow of 3.5 mL/min at room temperature until the conductivity is zero.
2. Adjust the pH of the product solution (from **Subheading 3.3.2.**) to 8.5.
3. Load the solution onto the column at a flow rate of 3.5 mL/min with a fraction size of 10.5 mL. During chromatography, all anions like HEPES, UMP, UDPGlc, and UDP replace chloride anions and are bound to the column.
4. Analyze the fractions by TLC.
5. Pool the fractions containing LacNAc, and adjust the pH to 7.0.
6. Analyze the pool by HPLC for LacNAc concentration.
7. Store the solution overnight at 4°C or over several days at -20°C until the next step.

3.2.3.4—***Gel Filtration***

1. Concentrate the pool (from **Subheading 3.3.3.3.**) at 20–25 mbar and 30–35°C to a volume of 9 mL in a rotary evaporator.
2. Equilibrate the Biogel P2 column with distilled water at a flow rate of 0.5 mL/min at 4°C.

3. Load the product solution onto the column at a flow rate of 0.5 mL/min and a fraction size of 3 mL.
4. Analyze the fractions by TLC.
5. Pool the fractions containing LacNAc.
6. Analyze the solution by HPLC for LacNAc concentration.
7. Freeze the solution at -20°C and lyophilize.
8. Solve 1 mg of the dry product in 1 mL distilled water and analyze by HPLC to determine the purity of LacNAc (approx 80%, *see* **Note** [5](#)).
9. The product can be stored at -20°C over several months.

4—

Notes

1. It is also possible to incubate the solution overnight and start the second batch after the ultrafiltration in the afternoon of the following day.
2. The consumption of alkaline phosphatase is minimized by using the repetitive batch-technique. Concentrate the solution by ultrafiltration to a volume of 5 or 10 mL, and start the second batch by addition of further product solution.
3. The synthesis can also be carried out in the absence of dUDP- and dTDP-6-deoxy-D-xylo-4-hexulose. In this case, 2.5 mL of fresh UDP-Glc-4'-epimerase solution instead of 2.5 mL dUDP- or dTDP-6-deoxy-D-xylo-4-hexulose has to be added in each batch.
4. If the yield of the synthesis should be maintained at >90%, fresh enzymes have to be added after two or three batches.
5. If the purity of the product is not good enough, some of the product isolation steps (anion-exchange chromatography, gel filtration, and ligand-exchange chromatography) must be repeated.

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20—

Production of Isomaltulose Using Immobilized Bacterial Cells

Peter S. J. Cheetham and Christopher Bucke

1—

Introduction.

The food industry is constantly seeking novel ingredients to improve existing products or to allow the introduction of new products. Such new materials must be safe, pure, and inexpensive, otherwise they will be unacceptable in concept. They must also have organoleptic and textural properties that make them acceptable to the consumer. Sucrose is an extremely valuable food ingredient: its best-known property is its sweetness but, compared with the “high intensity” sweeteners such as Aspartame® and saccharin, sucrose is not a very sweet material. In many cases the amount of sucrose required to provide the degree of sweetness required also provides significant bulk and texture to the product. Often, as in the cases of, for example, chocolate, cakes, and biscuits, consumers expect the bulk and texture provided by the sucrose. Public taste is changing, however, and the level of sweetness provided by the quantities of sucrose needed to give the desired bulk and texture are now excessive, at least to the tastes of many adults. Consequently there is a demand for ingredients that provide bulk and the correct texture to foodstuffs but less sweetness than sucrose. Several disaccharides provide lower levels of sweetness than sucrose and provide similar bulking properties but few of them have the appropriate textural properties. Isomaltulose is about one-third as sweet as sucrose but has a similar sweetness profile to that of sucrose. When used in products such as chocolate it provides a very similar texture to that given by sucrose but its lower sweetness does not mask the flavors of the other ingredients to the same extent. Isomaltulose is readily crystallized and is metabolized in a very similar way to sucrose. It shows a lower rate of release of monosaccharides into the blood.

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Therefore insulin release is correspondingly reduced compared with other sugars, creating the possibility of applications in diabetic and sports foods and drinks.

1.1—

Isomaltulose Production by Fermentation

Isomaltulose (known also as palatinose and lylose) is correctly 6-*O*- α -D-glucopyranosyl-D-fructofuranose. Chemical synthesis of isomaltulose is very difficult, but a small number of bacterial strains have proved capable of converting sucrose to isomaltulose. The South German Sugar Company was the first organization to produce isomaltulose, by fermentation using *Serratia plymuthica* (NCIB 8285) or a strain described as *Protaminobacter rubrum* (CBS 574.77). Cells were grown in an undefined medium containing 5% sucrose and other nutrients until a high-cell concentration was reached, after ~24 h. The cells were then used to convert a diluted refinery stream of sucrose (20% w/w), which was aerated and maintained at pH 7.0 until all the sucrose was converted to isomaltulose. The cells could be recovered using a jet separator and reused about six times, provided that sterile conditions were maintained. Isomaltulose may also be produced by a continuous fermentation process using sucrose solutions (25% w/w) at a dilution rate of ~0.1/h giving a steady state productivity of ~25 g isomaltulose/L·h. However, isomaltulose is now produced using immobilized cells.

1.2—

Advantages of Using Immobilized Cells

Immobilized cells have proved most effective in processes in which little or no oxygen is required and in which small molecules are interconverted using cell-bound biocatalysts. Much the best example is the conversion of glucose to high fructose syrup using glucose isomerase but many other examples can be provided, such as the production of L-aspartate and L-malate. Therefore the interconversion of sucrose and isomaltulose is an obvious candidate for the application of immobilized cell technology. Isomaltulose production occurs most efficiently when nongrowing cells are used, therefore cell production and product formation steps may be optimized individually, the cells produced optimally by conventional fermentation, then immobilized using an appropriate method. High densities of cells may be immobilized and used with concentrations of substrate which are inimical to cell growth. The energy requirements of packed-bed reactors containing immobilized cells are slight compared with fermenters which require agitation and aeration. Immobilized, nongrowing cells divert little substrate into cell material. Immobilized cell reactors may be used continuously, often for extended periods orders of magnitude longer than reactors using nonimmobilized cells.

1.3—

Immobilized Cell Reactor Development

The pioneering work on the use of immobilized cells in the production of isomaltulose (1,2) employed strains of *Erwinia rhapontici*. These were selected for their high productivity, as nongrowing cells, in converting sucrose to isomaltulose and their stability when used in an immobilized form. Cells were cultivated at 30°C in nondefined media containing sucrose, peptone, and beef extract. Small-scale cultures used 500-mL shake-flasks containing 200-mL of medium.

The enzyme activity proved to be stabilized by the presence of sucrose so it was advantageous to use a medium which was not sucrose limited. During cell growth, capsular polysaccharide was produced which interfered with the recovery of cells. This was apparently produced in response to high pO_2 values: *E. rhapontici* is a facultative anaerobe and it is plausible that the polysaccharide is produced when sucrose is available in order to impose a diffusional restriction on the rate of oxygen transfer from the bulk medium to the cells. Polysaccharide production appeared to be at the expense of isomaltulose synthesis since cells with high isomaltulose-producing activity were obtained from poorly agitated fermentations and vice versa. Once immobilized, the cells are in a relatively anaerobic environment due to the diffusion restrictions imposed so no further polysaccharide is produced and more isomaltulose forms.

Of many different immobilization methods tested, by far the most successful was entrapment of the whole cells in beads of calcium alginate (3). The beads were used most effectively in packed bed reactors. When very high initial sucrose concentrations (1.6 M) were employed in conditions where there was 99% conversion of sucrose to isomaltulose, the first order decay constant of enzyme activity was 1.95×10^{-2} per day, i.e., the operational half-life was 359 d.

Isomaltulose crystallizes readily, which provides a ready means of purification. The ease of production means that isomaltulose can be produced inexpensively: the South German Sugar Company produces several thousand tonnes of isomaltulose annually. Most of this is reduced by catalytic hydrogenation to produce Palatinit®, a 50–50 mixture of glucosyl mannitol and glucosyl sorbitol. This is as sweet as sucrose but is less metabolisable and less cariogenic than sucrose so finds use in “sugar-free, low-calorie” products.

This chapter describes the production of isomaltulose at the laboratory scale: for an account of the production of the sugar on a larger scale, see the article by Cheetham (4).

2—

Materials

1. Bacterial strains: apparently rather few microbes have the ability to convert sucrose to isomaltulose, the majority of them plant pathogens such as *Protaminobacter rubrum*, *Serratia plymuthica*, *S. marcescens*, *Erwinia carotovora*, and strains of

Erwinia rhapontici. Strains of the latter deposited in Britain at the National Collection of Plant Pathogenic Bacteria as NCPPB 1578, 139 and 1739 proved most satisfactory in our hands. The strain NCPPB 1578 is also deposited at the American Type Culture Collection under the Accession Number ATCC 29283; the methods described here were developed using this strain.

2. Sterilized growth medium containing 40 g/L sucrose, 10 g/L peptone and 4 g/L beef extract.
3. Sucrose: commercial granulated sugar is 99.95% pure and entirely satisfactory for this purpose.
4. Sodium alginate, 5% (w/v) solution in distilled water (see **Note 1**).
5. Phosphate-buffered saline.
6. A temperature-controlled orbital incubator.
7. 500-mL baffled shake flasks.
8. A centrifuge capable of 15,000*g*.
9. Syringes (at least 10 mL). Plastic disposable syringes are perfectly satisfactory, needles are not essential.
10. Jacketed column(s), e.g., 30 cm high × 5 cm diameter.
11. Peristaltic pumps.
12. A vacuum oven.

2.1—

Analytical Method

The progress of synthesis of isomaltulose, which is a reducing sugar, from sucrose may be followed using one of the well-known methods of measuring reducing sugar. The 2,4-dinitrosalicylate method is recommended.

3—

Methods.

3.1—

Cell Production.

1. Prepare and sterilize the growth medium containing 40 g/L sucrose, 10 g/L peptone, 4 g/L beef extract.
2. Dilute cells from a stock culture (maintained on nutrient agar slopes) with 10 mL of phosphate-buffered saline.
3. Inoculate 200-mL aliquots of growth medium in 500-mL baffled shake flasks with 0.1-mL aliquots of the cell suspension.
4. Incubate the inoculated flasks at 30°C for 70 h in an orbital incubator at 120 oscillations/min.
5. Harvest the cells by centrifugation at 15,000*g* for 10 min at 30°C. About 1 g packed wet cells/100 mL are obtained.

3.2—

Immobilization of Cells.

1. Prepare and sterilize a solution of sodium alginate, 5% (w/v) in distilled water. (see **Notes** [1](#) and [2](#)).
2. Suspend the harvested packed cells, as soon as possible after preparation, so as to form a 20% (wet w/v) suspension of cells in the sodium alginate solution.

3. Extrude the cell suspension dropwise from a height of 10 cm into a stirred solution of calcium chloride (0.1 M) containing 15% (w/v) sucrose, maintained at 30°C.
4. Stir the resulting pellets for 1 h.
5. Pack the pellets into a suitable jacketed column (e.g., 30 cm height, 5 cm diameter) maintained at 30°C.

3.3—

Production of Isomaltulose..

1. Prepare a solution of sucrose 55% (w/v) in distilled water, adjust to pH 7.0 using 1.0 M sodium hydroxide solution.
2. Pump the sucrose solution up the column, maintaining the temperature at 30°C, at a flow rate of 0.01 empty column volumes/h. At this flow rate a steady state is reached within 24 h and the conversion of sucrose to isomaltulose (see **Note 3**) approaches equilibrium. The productivity of the cells is about 0.2 g product/g wet cells/h. (see **Note 4**).
3. Evaporate the column eluate at 60°C to about 70% (w/v).
4. Cool, preferably with stirring. Small white crystals of isomaltulose form.
5. Collect the crystals by filtration or centrifugation.
6. Dry at 40°C in a vacuum oven at 700 mmHg. Typically the product contains about 95% isomaltulose (which crystallizes with one water of crystallization per molecule of isomaltulose).
7. Recrystallize three times from water to obtain the pure product. Dry the crystals as in **Step 6**.

4—

Notes

1. Many different grades of sodium alginate are available, all of which may be used satisfactorily for cell immobilization. The most effective is the high-guluronic alginate available from Merck-BDH.
2. Dissolving sodium alginate in water is not as easy as it might appear and it is worthwhile to devote time to the preparation of alginate solutions. Stir distilled water maintained at 40–50°C and add the solid sodium alginate carefully, avoiding the formation of large lumps of moistened material which is very difficult to dissolve further. Stir until dissolution is complete. It is not essential to sterilize the alginate solution before making immobilized cell preparations but if it is desired to do so autoclaving will lower the viscosity of the sodium alginate solution and increase its color. Neither of these phenomena affect the gelling properties of the alginate significantly.
3. On a small scale, alginate beads are prepared most conveniently manually using plastic syringes. Sterility is not essential so the easiest way of introducing the suspension of cells in alginate into the syringe is to remove the plunger and to pour the cell suspension into the syringe barrel, then replacing the plunger. Calcium alginate gel forms astonishingly rapidly and the syringe will block if the nozzle contacts the calcium chloride solution. If a syringe needle is used smaller beads may be produced. Several ingenious methods have been developed for the mass production of alginate beads (e.g., **ref 5**). The method described here was

scaled up using a stainless steel plate perforated with 36 holes of small diameter (0.5 mm) and coated with PTFA to facilitate extrusion. Cell slurry was pumped to this using a peristaltic pump capable of pumping 10 L/h. When preparing alginate beads it should not be forgotten that the liquid level in the gelling bath will increase so the nozzle(s) should be kept clear of the rising liquid surface.

4. Greater productivity may be obtained by increasing the flow rate of sucrose solution through the column: at 80% conversion the productivity is around 0.32 g product/g wet cells/h.
5. The other products contaminating the isomaltulose will be unchanged sucrose, glucose, fructose and trehalulose (1-*O*- α -D-glucopyranosyl- α -D-fructopyranose). The latter is an equilibrium-favored product of the action of the isomaltuloseproducing enzyme.

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21—**The Production of Mannitol by Fermentation**

Wim Soetaert, Petra T. Vanhooren, and Erick J. Vandamme

1—**Introduction****1.1—*****Chemical Properties of Mannitol***

D-Mannitol is a sugar alcohol with many applications in food, pharmaceuticals, medicine, and chemistry. Mannitol crystallizes in small white needles with a melting point of 165–170°C. Mannitol has a sweet cool taste owing to its high negative heat of solution (-121 kJ/kg). It is about half as sweet as sucrose. Mannitol has a low solubility in water of only 18% (w/v) at 25°C. Especially in alkaline solutions, it is a powerful sequestrant of metallic ions.

1.2—***Applications of Mannitol***

Mannitol finds its largest application as a food additive (E421). It is used as a sweet-tasting bodying and texturing agent. Mannitol reduces the crystallization tendency of sugars and is used as such to increase the shelf-life of foodstuffs.

Crystalline mannitol exhibits a very low hygroscopicity, making it useful in products that are stable at high humidity. It is used extensively in chewing gum, often in combination with other polyols, such as sorbitol and xylitol. Such chewing gum is noncariogenic and contains few calories (sugar-free chewing gum).

Because of its desirable properties, mannitol is commonly used in the pharmaceutical formulation of chewable tablets and granulated powders. It prevents moisture absorption from the air, exhibits excellent mechanical compressing properties, does not interact with the active components, and its sweet cool taste masks the unpleasant taste of many drugs (**1**).

In medicine, mannitol is used as a powerful osmotic diuretic for intoxication therapy. In many types of surgery, parenteral mannitol solutions are applied to prevent kidney failure. Mannitol is used in brain surgery to reduce cerebral

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edema. Mannitol hexantrate is a well-known vasodilator, used in the treatment of hypertension.

The complex of boric acid with mannitol is used in the production of dry electrolytic capacitors. It is used in chemistry for the production of resins and surfactants. Mannitol is used in analytical chemistry for boron determination. It is also used extensively in scientific research, especially in microbiology, where it is a classical carbon source for determinative purposes.

1.3—

Natural Occurrence

Mannitol is widely distributed in nature, even more extensively than its isomer sorbitol. It is frequently found in such plants as pumpkins, celery, onions, grasses, olives, mistletoe, and lichens. It often occurs in plant exudates, such as in manna, the dried exudate of the manna ash tree (*Fraxinus ornus*), from which its name was derived. This manna has been the commercial source of mannitol for many years and was produced in Sicily from the sap of *Fraxinus rotundifolia* (2–3).

Mannitol is commonly found in the mycelium of various fungi and is the main carbohydrate in mushrooms. It is also present in high levels in brown seaweeds where it is invariably the main product of photosynthesis (2). Some species (e.g., *Laminaria cloustoni*) can contain up to 20% mannitol.

Mannitol is also a common polyol in wine. Mainly in high-quality wines, mannitol contributes to the “full-bodiedness” of the wine (4). Especially in “noble rot” wines, high mannitol contents are found owing to the action of the *Botrytis* fungus involved.

Conflicting reports have been published concerning the metabolic fate of orally administered mannitol in the human body (5–9). Although some authors have published data suggesting that mannitol behaves as an inert substance in human metabolism, it is now generally agreed that mannitol is nontoxic, and is only partially metabolized by the human body because of the slow resorption, low solubility, and fast excretion via the urine. It is slowly resorbed in the digestive tract and therefore has laxative properties owing to fermentation in the colon. Its daily intake should not exceed 10–20 g/d.

1.4—

Microbial Formation of Mannitol

The production of mannitol by fermentation was attempted as early as 1931. Since mannitol is a common reserve product of many fungi and yeasts, of which some excrete it extracellularly, this property makes them suitable candidates for production purposes. Mannitol was produced from glucose by several *Aspergillus* sp., such as *Aspergillus elegans* and *Aspergillus nidulans*. A yield of 50%, based on glucose, could be reached (10). *Byssoschlamys fulva* also produced mannitol from glucose with a yield of about 30% (11). More recently,

several *Penicillium* strains were screened for their capacity to produce mannitol (12). All strains tested produced mannitol, among other polyols, such as glycerol, erythritol, and arabitol.

Also osmophilic yeasts are capable of producing and excreting mannitol (13–16). *Candida zeylanoides* was used for the production of erythritol and mannitol from alkanes (15). A high phosphate concentration caused a complete shift from erythritol to mannitol production in 52% yield. *Torulopsis mannitofaciens* was used for the production of mannitol from glycerol in over 50% yield (14).

Also, bacteria can produce mannitol from certain substrates. Especially heterofermentative lactic acid bacteria are known producers of mannitol from fructose. Heterofermentative lactobacilli, such as *Lactobacillus brevis* and *Lactobacillus buchneri*, form mannitol from fructose during silage fermentation. In addition *Leuconostoc mesenteroides* has been known for a long time to form mannitol when fermenting sucrose.

The biochemical basis of the mannitol synthesis by heterofermentative lactic acid bacteria is well known (17). Heterofermentative lactic acid bacteria use a combination of the hexosemonophosphate pathway and the phosphoketolase pathway for carbohydrate dissimilation. When the organism grows on glucose, this is converted to glucose-6-phosphate followed by dehydrogenation to 6-phosphogluconic acid, which is subsequently cleaved to ribulose-5-phosphate and carbon dioxide. Ribulose-5-phosphate is isomerized to xylulose-5-phosphate. The next step is catalyzed by phosphoketolase. The enzyme cleaves xylulose-5-phosphate into acetylphosphate and glyceraldehyde-3-phosphate, which is further converted into lactic acid. The acetylphosphate is normally reduced to ethanol, in order to maintain the redox balance in equilibrium. The organism is compelled to convert acetylphosphate to ethanol under anaerobic conditions, despite a considerable waste of energy (ATP) (Fig. 1). If an alternative electron acceptor, such as fructose, is present in the growth medium, the microorganism is capable of using this carbohydrate as an electron acceptor (Fig. 2). In this way, NAD(P) is regenerated by reducing fructose to mannitol. Acetylphosphate can then be converted to acetate, with the generation of an extra ATP. Overall, the microorganism obtains an extra ATP, which is the main driving force behind this conversion.

The enzyme mannitol dehydrogenase plays a key role in this conversion. It catalyzes the direct conversion of fructose into mannitol and vice versa:



The enzyme is cofactor-dependent; it requires the presence of reducing equivalents, such as NADH₂ or NADPH₂. Based on the hydrogen balance, the theoretical fermentation equation is then as follows:

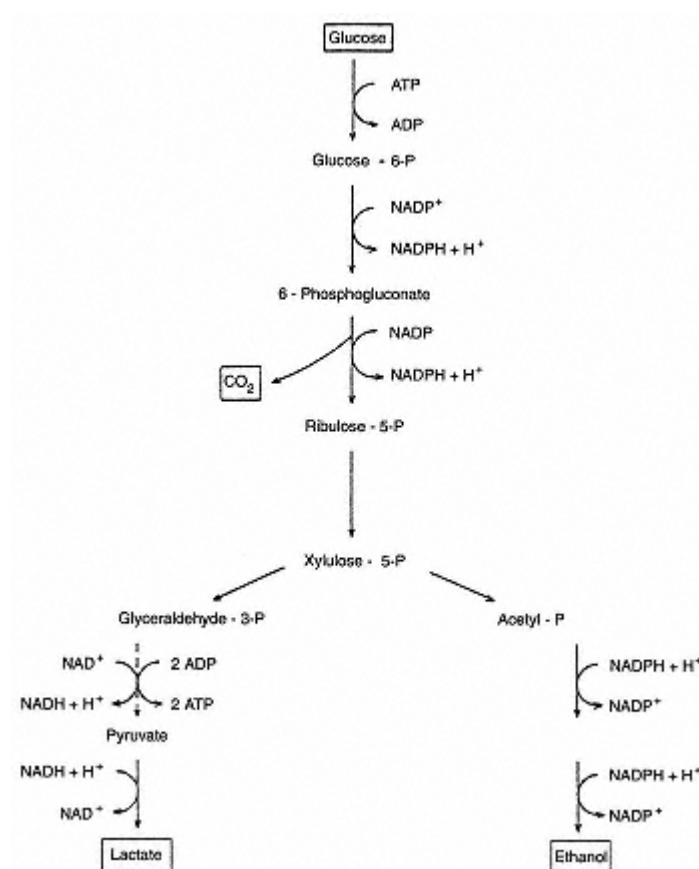
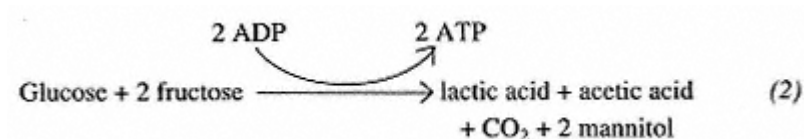


Fig. 1.
Heterofermentative conversion pathway of glucose.



This pathway is present in *L. mesenteroides* and in certain heterofermentative lactobacilli, such as in *L. brevis* and *L. buchneri*.

1.5—

Optimized Mannitol Fermentation Process.

Based on the above principle, a process for the microbial production of mannitol has been developed and optimized (18). In this fermentation process, *L. mesenteroides* is used to convert a mixture of glucose and fructose into mannitol. The process has been optimized to obtain a quantitative yield whereby mannitol is obtained in a concentration of 150 g/L and even higher.

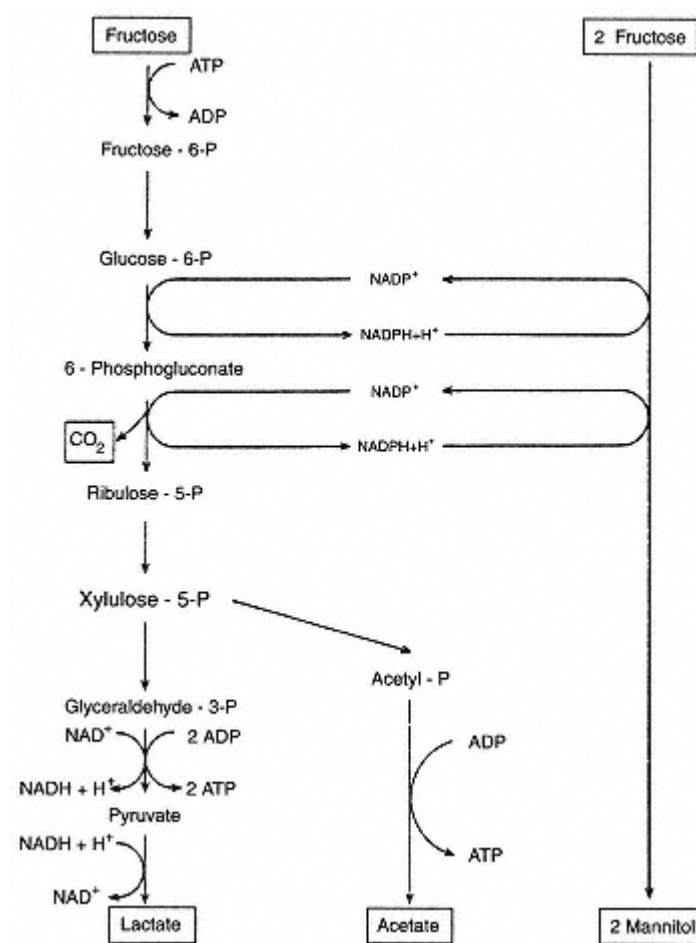


Fig. 2.

Heterofermentative conversion pathway of fructose into mannitol.

The fermentation procedure can either consist of a batch fermentation or a fed-batch procedure. A fed-batch procedure results in a faster conversion, but is more difficult to perform. During the conversion, glucose and fructose are converted simultaneously, whereby fructose is converted mainly into mannitol, and glucose is converted to acetic acid and lactic acid, supplying the reducing equivalents for the conversion of fructose to mannitol. The formation of organic acids during the fermentation will cause the pH to decrease down to a point where the fermentation will come to a standstill. Therefore, the formed organic acids must be neutralized with NaOH or CaCO_3 in order to keep the pH at an acceptable level so that the fermentation proceeds to completion. At the end of the fermentation, all fructose and glucose are converted, and the

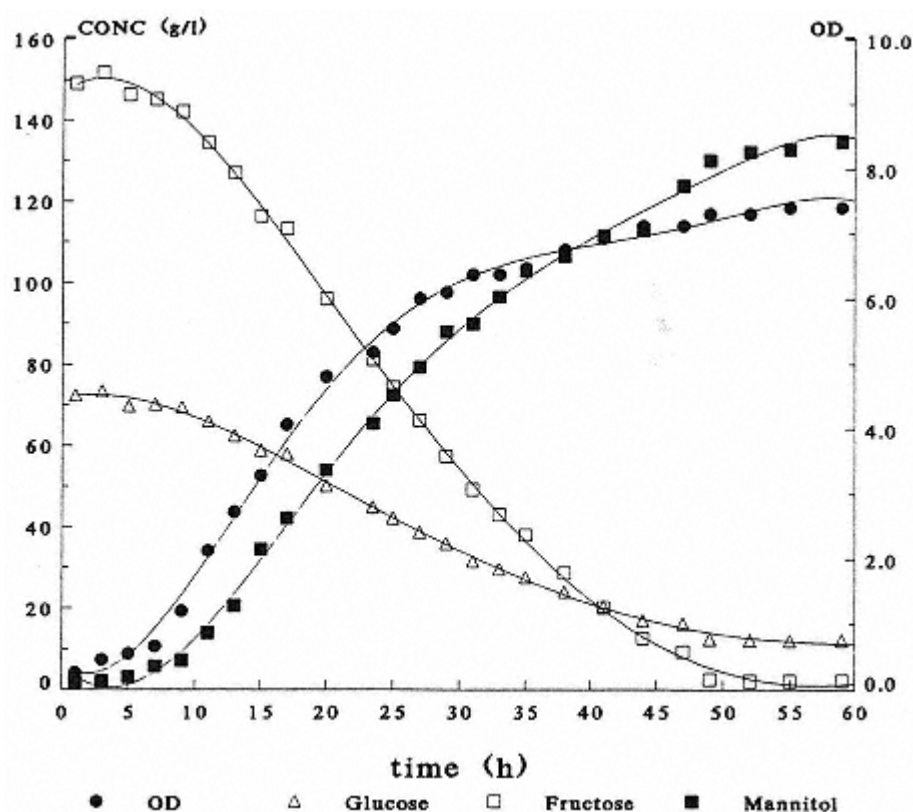


Fig. 3.
Batch fermentation profile for the microbial production of mannitol with *L. mesenteroides*.

mannitol concentration is at its maximum. The fermentation broth is stable at this point, since the cells are unable to metabolize the mannitol under the fermentation conditions. An example of a batch fermentation profile is shown in **Fig. 3**. It can be seen that glucose and fructose are used simultaneously, as is required for a good conversion. The glucose and fructose curves, however, intersect at a late point in the fermentation, which should not occur in the case of an ideal conversion. This observation reflects that the theoretical fermentation equation above has no practical validity. In practice, some fructose is also converted to organic acids instead of to mannitol, thereby taking over the role of glucose in the conversion. Part of the fructose is also used for the generation of reducing equivalents. Thus, the source of reducing equivalents in the fermentation is glucose as well as part of the fructose. The extent to which this undesired conversion occurs determines the conversion efficiency (moles of mannitol formed vs moles of fructose consumed). The conversion efficiency

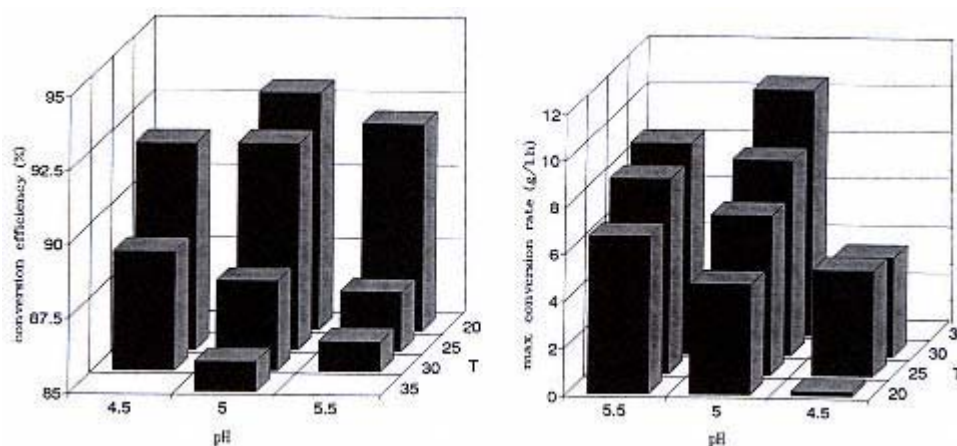


Fig. 4.

Conversion efficiency and conversion rate as a function of pH and temperature for batch mannitol fermentations with an initial substrate concentration of 50 g/L glucose and 100 g/L fructose.

appears to depend mainly on the pH and the temperature during the fermentation (**18**). Also, the conversion efficiency is inversely correlated with the conversion rate. The highest conversion rates are obtained by fermenting at higher pH values (5.5) and at higher temperatures (35°C). At a low temperature (20°C) and at low pH value (4.5), the conversion efficiency will increase to well over 90%, but the conversion rate becomes slower (**Fig. 4**). Depending on the type of fermentation procedure, an optimal compromise between conversion efficiency and conversion rate could be found at pH 5.0–5.5 and 20–25°C.

It is important to note that the biomass concentration is quite low. Typical biomass contents obtained at the end of the batch fermentations are 2–4 g/L, depending on the fermentation procedure. Consequently, the loss of substrate, which is incorporated in the bacterial cell mass, is low.

After the fermentation, the cells can be removed by centrifugation or by filtration. The mannitol can be obtained from the fermentation broth by vacuum evaporation followed by cooling crystallization, as will be described in **Subheading 3**. Alternatively, a higher mannitol yield can be obtained by first removing the organic acids from the broth by electrodialysis, followed by evaporation and cooling crystallization. The mannitol crystals are recovered from the mixture by centrifugation or filtration. Pure white needle-formed crystals are obtained with a purity of over 99%.

The practical procedure for fermentative production of mannitol is described in **Subheading 3**. It consists of a batch fermentation, whereby a mixture of glucose (75 g/L) and fructose (150 g/L) in a ratio of 1/2 is converted to mannitol, lactic acid, and acetic acid. The conversion efficiency of fructose into man-

nitrol is over 90%. The glucose is converted to organic acids. The lactic acid and acetic acid are neutralized during the fermentation by controlled addition of NaOH at a pH of 5.0. The fermentation is performed at 20°C and takes about 65 h to reach completion.

1.6—

Enzymatic Process for Mannitol Production

Purely enzymatic methods of producing mannitol based on the enzyme mannitol dehydrogenase have been developed. The cofactor dependence of the enzyme is the main limitation in this approach. The common way of circumventing this problem is by coupling the reaction with another enzyme reaction that is equally cofactor-dependent, but supplies reducing equivalents. Thus, glucose/fructose mixtures could be simultaneously converted into gluconic acid and mannitol using the enzymes mannitol dehydrogenase, glucose dehydrogenase, and a common cofactor NADH₂ (19). In this case, two interesting products are formed. The cofactor can also be continuously regenerated with formate dehydrogenase (20). This enzyme converts formate into carbon dioxide, thereby regenerating the cofactor. In this case, only one interesting product is formed, but this system has the advantage that formate is cheap and carbon dioxide does not cause any problems related to inactivation or inhibition of the enzymes involved. Moreover, carbon dioxide is easy to separate, leading the reaction to completion. In practice, several difficult problems remain to be addressed, such as retention of the cofactor in the reactor with special membranes, the strong product inhibition of the mannitol dehydrogenase, and its high K_m value for fructose.

1.7—

Chemical Process for Mannitol Production

Although the microbial production of mannitol has a long-standing history and recently a very efficient procedure has been developed with *L. mesenteroides*, mannitol is produced industrially by chemical means. Mannitol is produced by catalytic hydrogenation of fructose. The commercial production is performed by high-pressure hydrogenation (70–140 atm) of glucose/fructose mixtures in aqueous solution at high temperature (120–160°C) with Raney nickel as the catalyst (21). In the hydrogenation process, β -fructose is hydrogenated to mannitol, but α -fructose is hydrogenated to sorbitol. The selectivity of the nickel catalyst is such that about half of the fructose is converted to mannitol and half of it to sorbitol. The glucose is hydrogenated exclusively to sorbitol. As a consequence, the commercial production of mannitol is always accompanied by the production of sorbitol. Typically, the hydrogenation of a 50/50 fructose/glucose mixture results in a ca. 30/70 mixture of mannitol and sorbitol. Owing to the much lower solubility of mannitol as compared to sorbitol, pure mannitol can be obtained by fractional crystallization of the mixture. The filtrate can be sold as a sorbitol solution containing mannitol to some extent. Numerous process

improvements to increase the ratio of mannitol/sorbitol formation have been suggested or patented (22).

1.8—

Combination Synthesis of Mannitol

Combinations of enzymatic conversions and chemical hydrogenation to increase the ratio of mannitol/sorbitol have also been described. In one such production method, glucose is enzymatically oxidized with pyranose-2-oxidase to glucosone and subsequently catalytically hydrogenated to obtain a sorbitol/mannitol mixture, which predominantly contains mannitol (23). Recently, another chemo-enzymatic process has been patented. It consists of an enzymatic conversion of fructose to mannose with the enzyme mannose isomerase, followed by catalytic hydrogenation (24). Here also, the yield of mannitol vs sorbitol is increased markedly since mannose is converted exclusively into mannitol.

2—

Materials

2.1—

Microorganism

The microorganism used is *L. mesenteroides* ATCC 12291. It can be obtained from the American Type Culture Collection (12301 Parklane Drive, Rockville, MD 20852).

2.2—

Fermentation Media

2.2.1—

Sucrose Medium

This solid medium is used for culturing *L. mesenteroides* ATCC 12291. The medium consists of 10 g/L tryptone, 10 g/L yeast extract, 10 g/L K_2HPO_4 , 20 g/L sucrose, 10 mL/L vitamin/mineral solution, and 20 g/L agar at pH = 7.5. The pH of the solution is corrected at pH 7.5 by addition of NaOH or HCl prior to sterilization. Sterilization is performed at 121°C for 15 min, after which the filter-sterilized vitamin/mineral solution (see **Subheading 2.2.2.**) is added and Petri dishes are poured with the medium.

2.2.2.—

Vitamin/Mineral Solution

The vitamin/mineral solution is sterilized by filtration (0.2 μ m) and is added to the growth media after sterilization. The solution consists of 40 g/L $MgSO_4$, 1 g/L $FeSO_4 \cdot 7H_2O$, 20 g/L $MnSO_4 \cdot 4H_2O$, 1 g/L thiamin $\cdot$ HCl, 5 g/L ascorbic acid, and 3 g/L citric acid. The solution can be stored at 4°C for several months (see **Note 1**).

2.2.3—

Inoculation Medium.

The inoculation medium has the following composition: 10 g/L tryptone, 10 g/L yeast extract, 20 g/L K_2HPO_4 , 10 mL/L vitamin/mineral solution

(filter-sterilized), 40 g/L glucose, and 80 g/L fructose at pH 7.5. In order to avoid Maillard reaction during sterilization, the glucose and fructose are sterilized separately from the other medium components. In practice, for making 50 mL inoculum, two solutions are made. Solution A: 0.5 g yeast extract, 0.5 g tryptone, and 1 g K_2HPO_4 are dissolved in water up to 25 mL, after which the pH is corrected to 7.5 with NaOH or HCl. Solution B: 0.8 g glucose and 1.6 g fructose are dissolved in water up to 25 mL. Both solutions are sterilized separately (121°C/15 min) and poured together in a sterile manner after cooling to room temperature. Also 0.5 mL of filter-sterilized vitamin/mineral solution is then added to the mixture. The inoculation medium can be prepared in a 100-mL Erlenmeyer flask, equipped with a cotton plug.

2.2.4—

Fermentation Medium

The fermentation medium has the following composition: 10 g/L tryptone, 10 g/L yeast extract, 10 g/L K_2HPO_4 , 10 mL/L vitamin/mineral solution (filter-sterilized), 75 g/L glucose, and 150 g/L fructose at pH 7.0. In order to avoid Maillard reaction during sterilization, the glucose and fructose are sterilized separately from the other medium components. In practice, for making 1500 mL medium, two solutions are made. Solution A: 15 g yeast extract, 15 g tryptone, and 15 g K_2HPO_4 are dissolved in water up to 500 mL, after which the pH is adjusted to 7.5 with NaOH or HCl. Solution B consists of 112.5 g glucose and 225 g fructose, dissolved in water up to 1000 mL. Both solutions are sterilized separately (121°C/15 min). Solution B is best sterilized in the fermenter vessel itself, since it is the biggest volume and the most viscous solution. Solution A can be sterilized in an Erlenmeyer. After sterilization and cooling to room temperature, solution A and 15 mL of filter-sterilized vitamin/mineral solution are added in a sterile manner to the fermenter.

2.3—

Measurement of Bacterial Growth During the Fermentation

At regular time intervals, samples are taken from the fermentation broth. The optical density is measured as a quick means of following the bacterial biomass growth. To determine the optical density (OD), the sample is diluted five times with water, after which the extinction is measured in a 1-cm cuvet at 600 nm, using a spectrophotometer. The OD is then expressed as five times the measured extinction value.

2.4—

HPLC Measurement of the Carbohydrate Composition

At regular time intervals, samples are taken from the fermentation broth. The samples are cooled quickly to 4°C to stop the fermentation, and their carbohydrate content is determined afterwards by HPLC determination. Standard HPLC equipment can be used for measuring the carbohydrate content of the

samples. The separation column is an Aminex column HPX-87C, 300 × 7.8 mm (Bio-Rad), equipped with a precolumn Carbo-C microguard cartridge (Bio-Rad). The detector is a differential refractometer. The eluant consists of double-distilled or Milli-Q water, filtered (0.2 µm) and degassed under vacuum. The column is kept at 85°C, and the applied flow rate is 0.6 mL/min.

The fermentation samples are prepared in the following way. The broth is diluted five times with water, after which the cells are removed by centrifugation (4500 rpm for 10 min). Five milliliters of supernatant are pipeted into a tube, and 250 µL of a 20% glycerol solution are added and well mixed (internal standard). Subsequently, 1 g of ion-exchanger V (Merck, no. 4836) is added, and the contents are mixed for 1 h. After 1 h, the ion exchanger is removed with a paper filter, and the liquid is filtered through a microfilter (0.45 µm). The liquid is then injected into the injection loop of the HPLC (20 µL) and put on the column. The approximate retention times are: glucose 11 min 37 s; fructose 15 min 20 s; glycerol 18 min 13 s; mannitol 19 min 50 s.

As a standard, a carbohydrate solution is used containing 1% glucose, fructose, mannitol, and glycerol. The peak height (relative to glycerol) is taken as a measure for calculation. The response factors are determined at regular intervals to exclude daily variations. The fructose, mannitol, and glucose for the standard solution are dehydrated by drying at 60°C for 24 h under vacuum.

3—

Methods

3.1—

Inoculum Buildup for the Fermentation Process

1. The strain is cultivated by streak inoculation on the surface of a Petri dish with sucrose medium and incubating aerobically at 30°C. After 2–3 d of incubation, large mucous colonies will appear on the surface (see **Note 2**).
2. With a sterile needle, a single colony is picked up and transferred into 50 mL inoculation medium in a 100-mL Erlenmeyer equipped with a cotton plug.
3. The Erlenmeyer is incubated on a rotary shaker at 100 rpm at 30°C for 15 h. After 15 h of incubation, the growth should be clearly visible from the turbidity increase of the medium (see **Note 3**).
4. The inoculum is then inoculated in a sterile manner into the production fermenter.

3.2—

Production Fermentation

The production fermentation is performed in a 2-L fermenter, filled with 1500 mL of medium (see **Note 4**). The fermentation is performed semianaerobically (see **Note 5**), at 20°C, under slow agitation and at controlled pH conditions. The fermenter can be of any kind, provided it is equipped with an agitation and pH control system. The agitation system can be of any kind. The agitation speed is put at 200 rpm (1500-mL fermenter). The pH is continuously measured with a pH electrode and controlled during the fermentation by addition of a solution of 5 M NaOH at a pH of 5.0 (see **Note 6**).

After inoculation of the fermenter (50 mL), the pH of the broth will decrease fairly rapidly from ca. 7.0 down to 5.0 after ca. 10–15 h. At this point the pH controller will prohibit further pH decrease by controlled addition of NaOH solution. The fermentation will proceed, as can be measured by analysis of the samples taken at regular time intervals (see [Note 7](#)). The fermentation pattern is shown in **Fig. 3**. The glucose and fructose will gradually disappear, but the mannitol concentration will increase. After ca. 60–70 h of fermentation, the conversion should be completed, which can be seen by the complete disappearance of fructose, as determined by HPLC (see [Note 8](#)). Some remaining glucose can still be present at the end of the fermentation. It will slowly disappear on further fermentation, but this is not required. The mannitol concentration should be about 120–130 g/L at the end of the fermentation (see [Note 9](#)).

3.3—

Recovery of Mannitol from the Fermentation Broth

1. After the fermentation is completed, the microbial cells are removed from the fermentation broth by centrifugation at 5000 rpm for 30 min. The supernatant is a completely clear, yellow to slightly brown solution.
2. This solution is evaporated in a rotary evaporator at 40–50°C under vacuum (40 mbar) to a concentration of ca. 250 g/L mannitol.
3. The concentrated solution is then allowed to cool down to 20°C under gentle agitation.
4. At this point, ca. 1 g of finely ground crystalline mannitol is added to the broth to initiate the crystallization.
5. The solution is then placed in a cold room under slow stirring and is allowed to cool down to 0–5°C in a time span of ca. 15 h (overnight) ([Note 10](#)). During the cooling, part of the mannitol will crystallize from the solution as fine, brittle white needles.
6. About 24 h after crystallization starts, the crystals can be recovered from the solution by vacuum filtration on a Büchner filter equipped with a paper filter. After the mother liquor has been sucked through the crystal bed, 250 mL of ice-cold ethanol are poured over the crystal bed and sucked through, in order to remove the impurities from the crystals. The mannitol crystals are scraped from the paper filter and dried overnight under vacuum at room temperature.
7. Alternatively, instead of filtration, a basket centrifuge can be used to recover the crystals. The yield of the crystals is ca. 40% based on the mannitol present in the broth. The purity of the mannitol can be checked by HPLC and is generally over 99%.

4—

Notes

1. On addition of the filter-sterilized vitamin/mineral solution to the medium, a visible precipitation may occur, especially with the medium at higher pH values. This precipitation is normal and will dissolve when the pH of the medium decreases during the fermentation.

2. The colonies are rather large (5 mm) and mucous because of the formation of dextran, a biopolymer that is formed from sucrose by the microorganism. The optimal time of transfer from the Petri dish to the liquid medium is when the colonies are large, glossy, and mucus, typically 2–3 d after streaking out. On longer incubation, the colonies will tend to become less mucous and more opaque. If inoculated at that point in the liquid medium, a slow growth start is likely to occur.

3. If the growth (turbidity) is not visible, a longer time of incubation may be required. *See also* **Notes** [2](#) and [4](#).

4. The methodology described is for a small-scale experiment, whereby the production fermentation is performed in a fermenter with a working volume of 2 L, which is a classical laboratory fermenter size. The fermenter is filled with 1500 mL of medium, and an inoculum volume of 50 mL is used. If a larger or smaller size fermenter is to be used, then the used volumes of both the production fermentation and the inoculum can be increased or decreased linearly. When using a considerably larger inoculum volume, the time for obtaining a good inoculum growth may increase somewhat.

5. Semianaerobic fermentation conditions imply that the fermenter is not actively aerated, but also is not under a nitrogen atmosphere, as would be required for strictly anaerobic conditions. The broth is in contact with sterile air. It is essential that the fermenter is open to the environment (through a 0.45- μ m filter) in order to allow the escape of CO₂, which will be formed during the fermentation.

6. The NaOH need not be sterilized. The silicone tubes that are used for delivering the NaOH into the fermenter have to be sterilized together with the fermenter. About 200 mL of 5 M NaOH will be added during a fermentation with 1500 mL initial medium volume (when no samples are taken).

7. Samples can be taken at any interval of time. However, especially when using a smaller fermenter, such as the 2-L fermenter in this description, care must be taken to take small samples and not too many samples. Otherwise one might end up with a nearly empty fermenter by the time the fermentation comes to an end. Also, care must be taken that on emptying the fermenter in order to take samples, the agitation system and the pH electrode remain properly immersed in the broth, allowing their proper functioning.

8. Depending on conditions, a longer conversion time may be required to come to full conversion.

9. This corresponds to a conversion efficiency (moles of mannitol formed vs moles of fructose consumed) of ca. 95%. The lower concentration is the result of the dilution effect of the NaOH solution added.

10. The cooling rate can be accurately controlled at -1°C/h, as well as the agitation speed, which will all affect the crystal yield and crystal form. However, a carefully controlled crystallization is generally not required, since mannitol easily crystallizes from the solution.

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22—

The Production of 3-Keto-Derivatives of Disaccharides

Eberhard Stoppok and Klaus Buchholz

1—

Introduction

The microbial oxidation of disaccharides, and notably of sucrose, offers a route to selective synthesis of new products based on a range of industrially available sugars. Thus sucrose is produced in very large quantities with high purity and at a low price. Its specific properties, like high solubility in water, hydrophilicity, and biocompatibility would favor the application of derivatives in many sectors, like surfactants, cosmetics, and pharmaceuticals. Yet the difficulties of sucrose, and disaccharide chemistry in general, have limited their use in industrial production to very low quantities. These difficulties are due to the high functionality and similarity of the hydroxyl groups in synthetic routes, notably with sucrose as the basic material. Purity and homogeneity are required for the application of new products in the fields mentioned, and for new components in consumer commodities in general.

A key step in the synthesis and application of sucrose-based new chemicals may be its highly specific oxidation to 3-ketosucrose (α -D-ribo-hexapyranosyl-3-ulose-(1,2)- β -D-fructofuranoside) and ensuing technically feasible chemical routes to derivatives such as surfactants, monomers for polymerization (vinylsucrose) or conjugates e.g., with amino acids. Other disaccharides in turn, like lactose, maltose, and isomaltulose, which are also available on the industrial scale, can readily be oxidized to bifunctional carbonyl compounds with potential application as building blocks in composites or polycondensates. Furthermore, 2,3 enediols of 3-ketoglycosides have the ability to delay the oxidation and discoloration of aqueous foodstuffs and thus may be used as food antioxidants (**1**).

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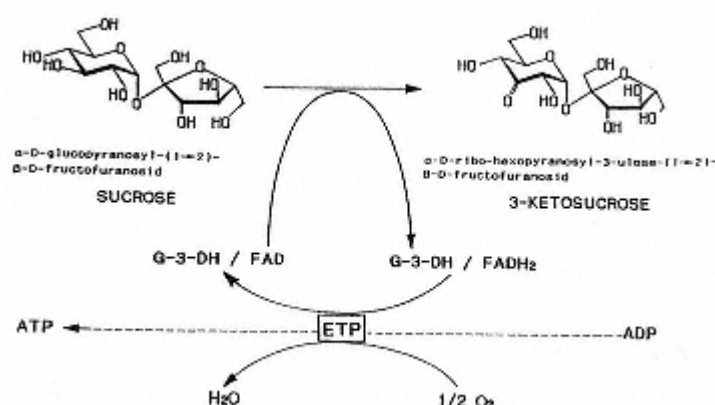


Fig. 1.

Oxidation of sucrose by *A. tumefaciens*: G-3-DH, glucoside 3-dehydrogenase; FAD, flavin adenine dinucleotide; ETP, electron transport pathway.

Bernaerts and de Ley (2) discovered the potential of *Agrobacterium tumefaciens* for the highly regioselective oxidation of several disaccharides. Further groups contributed insight into the microbiology and biochemistry of this transformation (3–5). The flavine adenine dinucleotide-dependent inducible enzyme, responsible for this oxidation reaction, has been identified as hexopyranoside cytochrome-c: oxidoreductase by Van Beeumen and De Ley (6). Hayano and Fukui (7) proposed use of the trivial name glucoside 3-dehydrogenase, which was also utilized by some authors. **Figure 1** shows the oxidation reaction with the above-mentioned enzyme exemplified with sucrose as the substrate.

Shortcomings cited in the literature were poor yields and low concentration of the products formed. Improved 3-ketosucrose concentrations in batch fermentations were recently obtained by Simiand et al. (8).

Our investigations aimed at reproducible and high yields, elevated product and low by-product concentrations, and transfer of the biotransformation to the pilot-scale level in order to utilize the potential of the reaction in follow-up synthetic routes (9–12). The kinetics of the reaction with respect to the oxygen concentration reveal an unconventional behavior (13). Essential further steps were simple and straightforward product recovery and purification and a more precise analytical photometric detection based on crystalline 3-ketosucrose as well as NMR data for several 3-keto-disaccharides. The range of disaccharides thus prepared, including novel products, is presented in **Fig. 2**.

We present here the production of 3-ketosucrose in a two-step reaction without and with sucrose fed batch. In a further step, the 3-ketosdisaccharide produced during the fermentation and ketodisaccharide produced from the resting

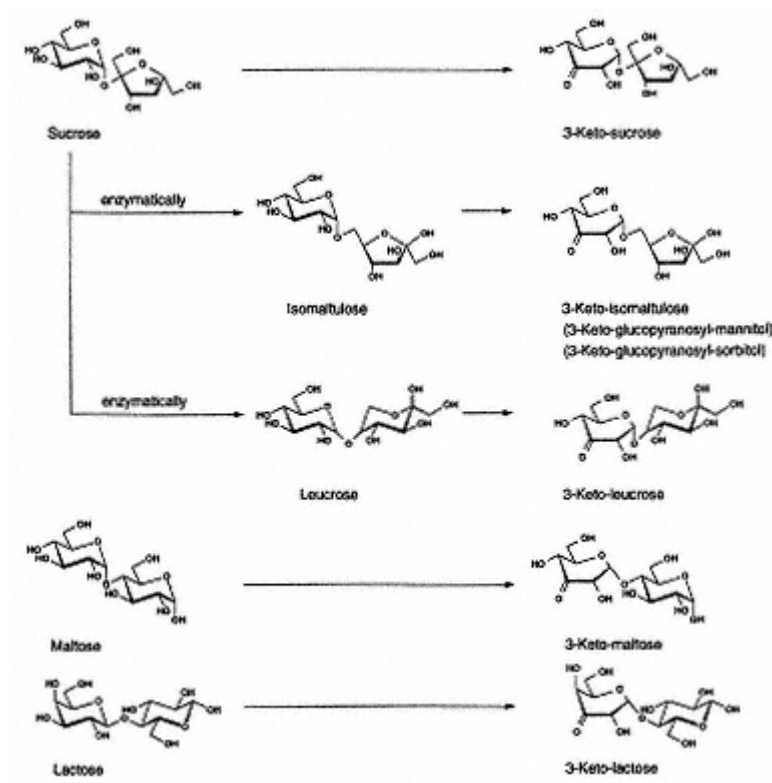


Fig. 2.

Formation of 3-keto-disaccharides from different substrates by *A. tumefaciens*.

cells can be combined in order to maximize the substrate based product yield. Furthermore, an optimized approach is fed batch in order to improve the yield in enzymatically active cells. Finally the oxidation of isomaltulose will be mentioned as an example of other 3-keto-disaccharides which can be obtained in 80% yield. In a final section, we briefly point out chemical routes that have been elaborated for the synthesis of a rather broad range of products with application potential, most of them carried out in aqueous solution, without protecting groups and with standard technology ready for scale-up.

2—

Materials

1. Micro-organisms: Various strains of *Agrobacterium tumefaciens* are able to oxidize sucrose to 3-ketosucrose. Among all isolates tested strain NCPPB 396 (National Collection of Plant Pathogenic Bacteria, Harpenden, UK) was selected, as it had been shown to give the best yields of ketosucrose formation.

2. Cultivation: The culture is maintained on agar slants with a medium consisting of 5 g/L peptone, 3 g/L meat extract, and 15 g/L agar-agar (deionized water).

For shaking cultures and precultures for fermentations, the culture medium of Kurowski and Darbyshire (5) was modified and consists of 0.9 g/L urea, 0.5 g/L yeast, 0.15 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.16 g/L citric acid, 5.4 g/L KH_2PO_4 , 10.8 g/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 20 g/L sucrose. The pH of the medium is adjusted to 7.0 prior to sterilization.

3. Fermentations: For fermentations, the concentration of KH_2PO_4 and Na_2HPO_4 is reduced to 1/5 of that of shaking cultures. Fermentations are carried out in a 20-L Biostat E with a working volume of 14 L (or alternatively, in a 70-L bioreactor Biostat U with a working volume of 50 L), both from B. Braun Biotech, Melsungen. Both reactors are equipped with an air control device that opens or closes an air control valve and increases or decreases the stirrer speed, thus controlling the air supply to the reactors.

Samples are taken periodically from the reactors and centrifuged immediately. The supernatant fluids are filtered through a 0.45- μm filter and assayed for sugars. If necessary, the samples prepared in this way are stored at -20°C for subsequent analysis.

4. Dehydrogenase activity: In order to control the activity of the cells during the cultivation, the cells derived from the samples are washed twice with 0.1 M phosphate-buffer, pH 7.0. In a standard procedure these resting cells are incubated at 27°C with a cell mass concentration of 50 g /L BWW (bacterial wet weight) and 50 g/L sucrose at pH 7.0 in 0.1 M phosphate buffer. This reaction is performed in 100- mL Erlenmeyer flasks filled with 40 mL of the reaction mixture (containing 2 g cells and 2 g disaccharide) for 20 h. The oxidation reaction does not proceed optimally under conditions of low oxygen supply, but very useful information is nevertheless obtained concerning the state of activity of the cells at different cultivation times.

5. Reaction mixture for the oxidation: For the oxidation of disaccharides to 3-keto-disaccharides, cells are harvested when the activity of the hexopyranoside c:oxidoreductase is maximal (20 h with Erlenmeyer flasks, 15–20 h in bioreactors). Resting cells obtained from the fermentation are incubated at a temperature of 27°C with a cell mass concentration of 25–100 g/L bacterial wet weight and 25–100 g/L disaccharide (in water or buffer) in a stirred and aerated reactor. Hence the oxidation with resting cells can be performed a bioreactor, provided sufficient cell mass is available. The pH is adjusted to 7.0 with NaOH at the start of the reaction if the reaction starts in water.

6. Storage: For the storage of resting cells, a concentrated cell suspension is stripped with nitrogen to prevent loss of activity due to oxygen and kept at 4°C until use (max. 3 d). If cells are frozen for longer storage periods they must be thawed quickly at 27°C in a water bath.

7. Analytical method: 3-keto-disaccharides, disaccharides, and fructose are determined by HPLC (RI detector) with a Merck Lichrosphere NH_2 (5 μm) column (4 $\times$ 250 mm, precolumn 4 $\times$ 4 mm, column temperature 22°C). The injection volume is 20 μL and elution is at 0.8 mL/min with acetonitrile/water (80/20) as the solvent system. For quick determinations, 3-ketosucrose is also determined by a photometric method based on that described by Fukui and Hayano (14).

A molar extinction coefficient at 340 nm of $E = 6.5 \times 10^3/M/cm$ was used by these authors. In contrast to these results, we determined a molar extinction coefficient of $E = 4.3 \times 10^3/M/cm$ with pure, crystalline 3-ketosucrose. This coefficient gives results comparable to those determined by HPLC and is used here.

3—

Methods

3.1—

Production of 3-Ketosucrose in a Two-Step Reaction

3-Keto-sugars are excreted into the culture medium in batch fermentations. Therefore, the production of 3-keto-disaccharides can be performed directly by isolation of 3-keto-sugars from the culture medium. As they are not easily separated from this medium, a two-step procedure with the use of resting cells was preferred. Resting cells with a high enzyme activity provide the advantage of achieving higher product yields via use of higher cell densities in the reaction mixture. Although different new 3-keto-derivatives were produced by *Agrobacterium tumefaciens*, we will exemplify the production of 3-keto-disaccharide with sucrose as the substrate investigated most fully.

3.2—

Batch Fermentation

In a first step, cell mass with high dehydrogenase activity is produced by fermentation. Cells are then harvested at the point of maximum enzyme activity and, in a second step, these resting cells are used for the oxidation of a sugar to its 3-keto-derivative. Although the range of some standard fermentation parameters may vary slightly without major influence on the enzyme activity required for the production of 3-ketosucrose, we will give exact fermentation data as an example in this chapter. Alternative parameters are described in **Notes** [1](#)–[3](#).

3.3—

Cultivation and Inoculation

1. Carry out all cultivations at a temperature of 27°C.
2. For the inoculation of precultures, transfer fresh cells from agar slants to 100 mL culture medium in 500-mL Erlenmeyer flasks.
3. Incubate the flasks for 40–48 h on a rotary shaker at 100 rpm.
4. For the following main culture (= fermentor preculture), inoculate 2000-mL Erlenmeyer flasks each filled with 500 mL (800 mL) of culture medium with 5 mL (8 mL) of the preculture and incubate for 20 h.
5. Transfer an amount of 1500 mL (3500 mL) medium from these cultures to an inoculation set and utilize it for the inoculation of 12.5 L (46.5 L) medium in the bioreactor (values in brackets for 50-L fermentations).
6. Aerate the bioreactor for the first 4 h by a rate of 0.1 vvm (vvm: volume air/cultivation volume per minute), increase then to 0.5 vvm, and set the stirrer speed to 400 rpm (180 rpm). Under these conditions a sufficient oxygen supply is provided for the growth and a decrease below 10% pO₂ can be avoided (see **Note** [2](#)).

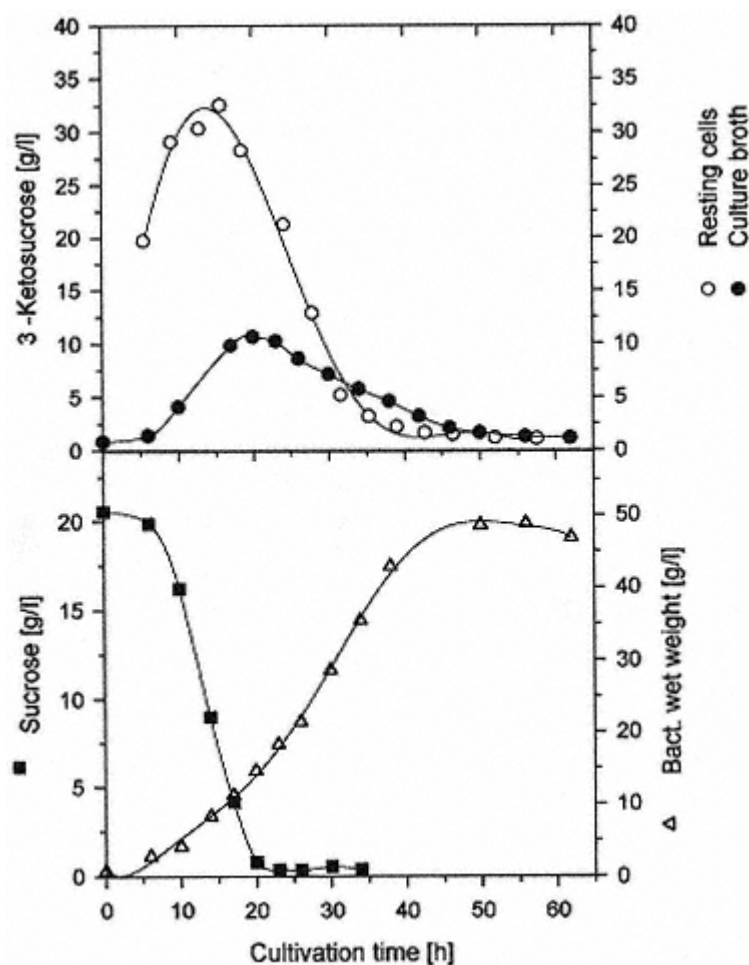


Fig. 3.
Time-course of a batch fermentation of *A. tumefaciens*.

7. Set the pH at the start of fermentation to 7.4. After a slight increase to 7.5 in the first 3 h of the cultivation, it may decrease finally to 6.7 at the end of the fermentation.

The typical fermentation run of the fermentation described above is shown in **Fig. 3**. The maximum of 3-ketosucrose in the culture medium is reached after a cultivation time of 20 h. In all fermentations, the 3-ketosucrose formed in a bioreactor or in a reaction mixture with resting cells the 3-ketosucrose concentration is transitory and decreases continuously after the maximum is reached. This decrease is explained by the hydrolytic activity of β -galactosidase and α -glucosidase and may be reduced by cultivation of *Agrobacterium* with lactose as carbon source (15). The activity of the resting cells is maximal

at an early stage of the growth, e. g., after 15–20 h of cultivation. Therefore cells for the production of 3-keto-derivatives in a subsequent step must be harvested at this point of maximal enzymatic activity. The cell mass concentration at the harvest time is at 10 g/L bacterial wet weight at this point of cultivation, which provides a total amount of 140 g resting cells for the production of 3-keto-disaccharide. In order to produce higher amounts of 3-keto-disaccharides, an increased amount of cell mass from a fermentation is desirable.

3.4—

Fed Batch Fermentation

The problem of obtaining higher amounts of cell mass with high dehydrogenase activity was solved by a fed batch of sucrose and is exemplified here for a 14- L fermentation.

1. Prepare a batch fermentation as described in **Subheadings 2.1.–2.3.** and **3.3.**
2. Sterilize 280 g of sucrose in a volume of 500 mL water in an inoculation set.
3. Add the sucrose aseptically to the fermentor when the sucrose in the bioreactor is nearly consumed, e.g., after 15 h of cultivation.
4. When the addition is performed by a sterile pump tube, it should be added within 20 min or even more rapidly (see **Note 1**).

It should be noted that the latest time for sucrose fed batch is at the point of sucrose exhaustion or even some time before, normally in the range of 13–19 h of cultivation time. When sucrose fed batch is performed after the complete consumption of sucrose the activity of the cells will not reach a clear second maximum. With sucrose fed batch, a cell mass concentration of nearly 40 g/L is reached at the point of maximal dehydrogenase activity. In comparison to a normal batch fermentation, a nearly fourfold amount of cell mass is provided for the production of 3-keto-disaccharides (**Fig. 4**).

3.5—

Production of 3-KS with Resting Cells.

1. Harvest the active cells of a fermentation or fed batch fermentation at the point of maximum enzyme activity.
2. Wash the cells as described in **Subheading 2.4**. Washing the cells may facilitate subsequent isolation procedures but is not necessary for the oxidation reaction.
3. Perform the oxidation of sucrose with resting cells and substrate in water in an aerated reactor (described in **Subheading 2.5**.)
4. Use at least 25 g/L cell mass and an initial substrate concentration of 25 g/L to provide high product formation during the reaction (see **Note 4**).
5. Adjust the pH to 7.0 at the start of the oxidation (see **Notes 3** and **5**).
6. Aerate the reactor and avoid oxygen limitation at any reaction time, e.g., by measurement of the oxygen partial pressure in the reaction mixture (see **Note 2**).
7. Avoid a pH decrease below 6.0 and correct with 10% NaOH if necessary.

8. Measure the 3-ketosucrose content periodically and stop the reaction when a maximum of 3-ketosucrose is reached.

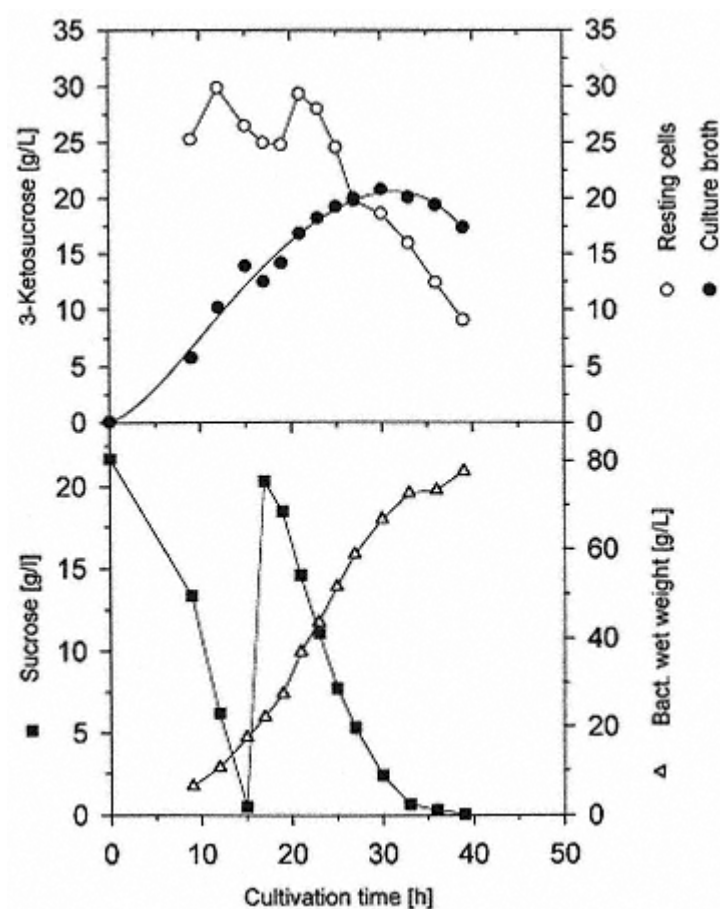


Fig. 4.
Time-course of a fed batch fermentation of *A. tumefaciens*.

In **Fig. 5** a typical time-course of sucrose oxidation with resting cells is shown. The product formation reaches a maximum and then gradually decreases. The sum of sucrose and 3-ketosucrose begins to decrease some time sooner as the maximum of 3-ketosucrose is reached.

Conflicting results have been published on the appropriate pH during 3-ketosucrose formation (**3,4,16**). High yields of 3-ketosucrose are observed at pH values of 7.0–7.6 at relatively short incubation times of 7.5 and 5.0 h, respectively (**10**). Yields of 68 % are obtained in water without buffer when the reactions are prolonged over 4 h to a complete turnover of the substrate. The control of complete substrate turnover is recommended in order to facilitate product recovery. With leucrose and isomaltulose as substrates obviously higher yields can be obtained (**10**).

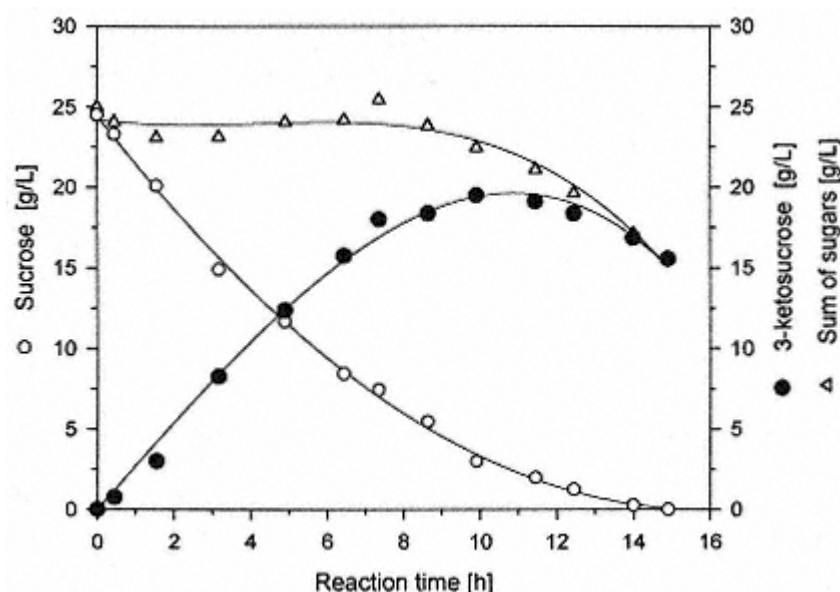


Fig. 5.

Formation of 3-ketosucrose with resting cells of *A. tumefaciens* in aerated reactors. The biocatalyst concentration (cell wet weight) and sucrose concentration were 25 g/L each. The aeration rate was 6.0 vm at pH 7.0.

The aeration rate must be adjusted to maintain at least 10% of oxygen saturation in the reaction mixture, so control of the oxygen concentration is recommended.

Concentration of the solution in vacuum is advisable, followed by membrane filtration using a cutoff of 0.45 μm in order to remove polysaccharides. The crude product can be obtained by freeze drying yielding 91% 3-ketosucrose. If the substrate is converted accurately during the oxidation reaction, sucrose and fructose occur in concentrations below 1% (water content 7% of dry matter).

The product solution can also be stored in the frozen state (in portions for optimal further handling). The crude product can be used for further technically established reactions, like hydrogenation to allosucrose (17).

Pure 3-ketosucrose (as well as other 3-keto-disaccharides) is obtained by column chromatography with a conventional Ca ion exchanger in water.

The product can finally be obtained by freeze drying as dry matter. Further chromatography gives a highly purified product for analytical purposes (18) (see Notes 6–8).

3.6—

Other 3-Ketodisaccharides

Similar to sucrose, other disaccharides and derivatives of disaccharides, like the sugar alcohols obtained by catalytic reduction of disaccharides, can be oxidized to give i.g. 3-keto -maltose, -lactose, -isomaltulose, -leucrose (10).

From the reduced isomaltulose (glucopyranosyl- α -1,6-maltitol as one of the isomers obtained) 3-keto-glucopyranosyl α -1,6-maltitol is obtained and even 2-amino- glucopyranosyl mannitol (α -D-glucopyranosyl-(1,6) D-2-amino-2-desoxy-mannitol) was found to be a substrate for the oxidation with *Agro-bacterium tumefaciens*(18,19).

The yields from several substrates are usually somewhat higher than from sucrose. Thus yields of other 3-keto-disaccharides after oxidation of the disaccharide by resting cells were 63% for 3-ketoleucrose and 72–90% for 3-keto-isomaltulose (10,18).

3.7—

Derivatives from 3-Ketosucrose

The main interest in the derivatives obtained via the oxidation described here is their function as disaccharide building block. The chemical synthesis of regioselective sucrose derivatives is laborious as mentioned before. 3-Ketosucrose opens the route to a range of regio- and stereoregular sucrose derivatives:

1. The catalytic hydrogenation with Raney Nickel selectively gives D-Allopyranosyl- α -1,2 β -D-fructofuranoside with more than 90% yield (17,20). From this disaccharide allose can be obtained in high yield by hydrolysis via classical protonated ion exchangers or via invertase and subsequent ion-exchange chromatography (20). The disaccharide as well the monosaccharide mixture and allose are potential sweeteners (21). Using leucrose, the same procedure yields new disaccharides: D-Allosyl α -1,5 D-mannitol, and -D-sorbitol (22).
2. Via the classical route of reductive amination 3-Deoxy-3-aminosucrose as a versatile building block is obtained (12). It offers the way to a range of substitution reactions and products of interest for technical application.
3. Surfactants are obtained by substitution with fatty acid esters via transesterification, e.g., with methyl laureate (12).
4. Building blocks for polymerization can be synthesized by introduction of acrylic type side chains with methacrylic anhydride, or with isocyanatoacrylates (12,23).
5. Aminoacyl- and peptide-conjugates are obtained through conventional peptide synthesis with activated and protected amino acids. Deprotection gives glyco-derivatives with unconventional substitution pattern: 3-(aminoacyl-amino-) allosaccharides (24,25).
6. The cyanhydrin, introducing a C-C-bound side chain, is obtained via the cyanhydrin reaction in neutral aqueous solution with good yield (20).

All these routes essentially proceed with high regioselectivity and mostly also with high stereoselectivity in aqueous solution without protecting groups; the advantages of the derivatization of sucrose via this microbial oxidation are obvious.

7. C-C-bound derivatives can also be obtained by Grignard reactions, however, in organic solvents with the protected 3-ketosucrose. Silylation with chlorotrimethylsilane in pyridine proved to offer a rather elegant access to full protection of the hydroxyl groups. Allyl as well as alkyl (up to dodecyl) side chains can subse-

quently introduced by Grignard reactions. Deprotection yields the corresponding sucrose derivatives, where the dodecyl derivative exhibits good surface active properties (12).

4—

Notes

4.1—

Cultivation

1. Fed batch: If fed batch of substrate is performed, it should be added within minutes with a sterile pump or even directly into the fermentor by an inoculation set. Otherwise, if the fed batch of sucrose is extended to some hours, no explicit second maximum of enzyme activity will become visible.
2. Aeration: Normally the aeration of the fermentation is achieved by using a constant aeration rate of 0.5 vvm. This may be too low during the exponential growth phase if higher substrate concentrations are used. Hence the application of an air-control device, which opens or closes an air control valve and increases or decreases the stirrer speed, can provide sufficient aeration at any cultivation time thus providing sufficient dehydrogenase activity. Constant aeration is performed at 20% pO₂; use of higher aeration may cause viscosity by polysaccharide formation.
3. Nitrogen source: Studies with various nitrogen sources revealed that urea was superior to other nitrogen sources, but an increase of the substrate concentration in cultivations requires also use of higher urea concentration, thus leading to an evident pH increase. Hence, precautions must be taken to avoid pH shifts which may decrease the yield of 3-keto-disaccharides.

4.2—

Resting Cells

4. Substrate concentration and aeration: During the oxidation reaction a limitation of the reaction velocity must be avoided. The concentration of substrates and resting cells can be favored from 25 g/L up to 100 g/L each, if the aeration rate is adjusted correspondingly.

Therefore an aeration with constant pO₂ at 20% is preferably used, providing sufficient aeration at any reaction time. At distinctly higher aeration rates the formation of polysaccharides can increase causing viscous reaction mixtures.

5. pH: Performance of the oxidation in a reaction mixture with water at an initial pH of 7.0 is recommended, although a reaction in buffer at the same pH will give higher initial reaction rates. However, a higher final yield of 3-ketosucrose is achieved when the pH decreases slightly during the reaction, as 3-ketosucrose is more stable under these conditions (10).

4.3—

Product Purification

6. If necessary, insoluble and high-molecular-weight by-products (low concentrations in general) can be removed prior to ion exchange chromatography by centrifugation and membrane filtration (pore width 0.45 µm.). Gel filtration with Sephacryl S-200 HR (Pharmacia Sweden) or with Fractogel TSK HW 40 (Merck, Germany) has been used in order to remove polysaccharides (22). Precipitation with alcohol removes polysaccharides.

7. Mono- and disaccharide by products (fructose and sucrose mainly) can be removed by ion exchange chromatography with conventional Ca ion exchangers, e.g., Amberlite CG 120 II (from Serva, Germany) in water; a column of 3×100 cm is loaded with 0.7 g of product in 1.5 mL, and elution proceeds with 3 mL/min at 5°C. A purified product is thus obtained (**26**).

8. Highly purified 3-keto-disaccharides can be obtained by centrifugation, membrane filtration, or gel chromatography as described before and further preparative HPLC on Lichrospher NH2 (7 μ m, column 32×250 mm, Merck, Germany) in acetonitrile/water (80:20, v/v) at room temperature. For the preparative HPLC a 2- mL sample containing 100–120 mg of 3-ketopalatinose is injected using an elution rate of rate of 35 mL/min (**18**), freeze drying of the product is followed.

5—

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23—

Enzymatic Synthesis of α -Butylglucoside in a Biphasic Butanol-Water System Using the α -Transglucosidase from *Aspergillus niger*

Marie-Pierre Bousquet, René-Marc Willemot, Pierre F. Monsan, François Paul, and Emmanuel Boures

1—

Introduction.

The multiple functional groups and stereocenters present in carbohydrates make them quite interesting targets for the organic chemist. Moreover, the use of renewable raw materials, such as corn starch, for synthesis reactions, is in accordance with environmental concerns.

Alkylglucosides are obtained by alcoholysis of starch hydrolysates **(1,2)**. They are a group of nonionic surfactants and exhibit an advantageous biodegradability **(3)**. They are applied in detergents, food, and pharmaceutical fields **(4)**. In addition, they can be used as substrates for the production of acylglucosides **(5–8)**, which are also surfactants. Enzymatic glycosylation can be a complementary tool to the chemical approach **(9)** since it is stereoselective and the product can be obtained by a one-step reaction **(6,10,11)**.

For alkylglycoside synthesis, two types of enzymes are available: glycosylhydrolases (EC 3.2.1) and glycosyltransferases (EC 2.4.1). Glycosylhydrolases require low water activity media to reverse hydrolysis in favor of synthesis. This can be achieved by using either a high substrate concentration **(12)** or an organic medium **(10,11,13)**. Glycosyltransferases, in contrast to glycosylhydrolases, can work in dilute solution and have been used for alkylglycoside synthesis in biphasic systems **(6)**. In such systems, yields depend on the solubility of the alcohol in the aqueous phase, where the enzymatic reaction occurs. Therefore, temperature is an important parameter, and thermostable transglucosidases are of particular interest. Enzyme denaturation happens mainly at the

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alcohol/water interface (**6,14–16**). It can be limited by entrapping the enzyme into calcium alginate beads, which significantly stabilize the protein (**6,14**).

The continuous production of α -butylglucoside in a biphasic butanol-water system is described using α -transglucosidase from *Aspergillus niger*. The enzymatic aqueous phase is entrapped into calcium alginate beads, whereas the butanol phase circulates upward in the column. The high porosity of alginate polymers usually limits their use to whole cells. Indeed, even large molecules, like proteins, diffuse out of the structure (**17**). In the system here described, α -transglucosidase can partition between the beads and the interstitial aqueous liquid, but its insolubility in butanol makes it impossible for the enzyme to leak out of the aqueous phase. On the other hand, α -butylglucoside, which can distribute between the two phases, is removed from the aqueous stationary phase and recovered at the outlet of the reactor. Since α -butylglucoside is also a substrate for the α -transglucosidase, its continuous extraction prevents its reuse by the enzyme. Moreover, the equilibrium obtained in a batch reactor can be displaced, and an efficient synthesis process can be operated.

2—

Materials

1. Sodium alginate solution: Prepare several hours before required. A solution of alginate 1.5% (w/w) is prepared by dissolving solid sodium alginate (SG300; Systems Bio-Industries, Carentan, France) in buffer solution appropriate for the enzyme (sodium acetate buffer, 50 mM, pH 5.0) (see **Note 1**). The dissolving process is slow and normally requires stirring for up to 10 h (see **Note 2**). The solution may be stored at 4°C and should be prepared every week.
2. Calcium chloride (CaCl_2 , $6\text{H}_2\text{O}$, Prolabo) solution: Prepare a solution of 0.15 M in buffer solution appropriate for the enzyme. The volume of this solution should be about 10 times the volume of sodium alginate solution. Store at 4°C, and prepare every week.
3. Enzyme buffer: 50 mM sodium acetate buffer, pH 5.0. Store at 4°C, and prepare every week.
4. Butanol (Normapur).
5. α -transglucosidase from *A. niger* (Amano, Japan): dilute 15 times in enzyme buffer to reach a working activity of 400 U/mL (see **Notes 3** and **4**). Add maltose (40%, w/v).
6. Reactor: The reactor is a tubular glass column of a working capacity of 10–100 mL (**Fig. 1**). It should be jacketed allowing the stationary phase to be thermostated.

3—

Methods

1. Transfer the alginate mixture to a dropping device, such as a syringe (10–100 mL), that has a means of flow control (see **Note 5**).
2. Place a beaker containing 0.1–1 L of calcium chloride solution (about 10 times the volume of the alginate solution) below the dropping device and magnetically stir the solution to produce a light vortex.

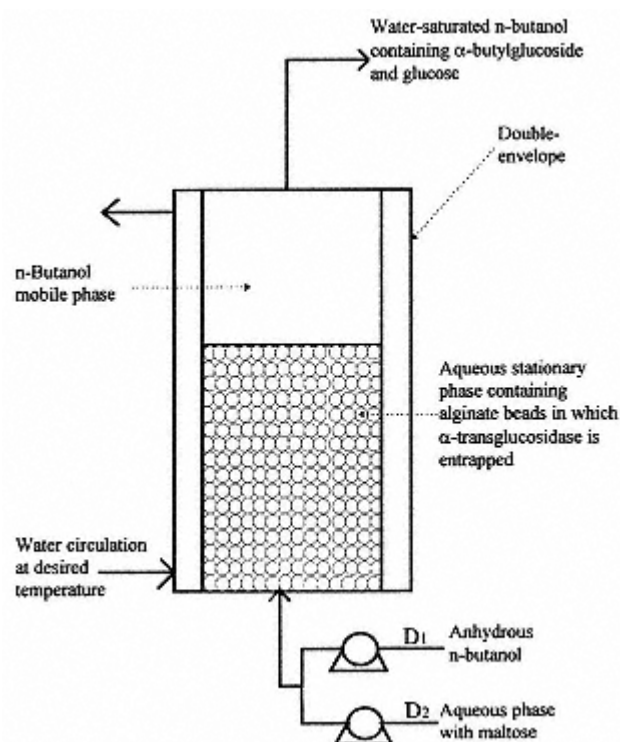


Fig. 1.
Continuous reactor for α -butylglucoside production.

3. Adjust the flow control to allow dropwise flow from a height of about 10 cm into the calcium chloride solution (see **Note 6**).
4. Allow the flow to continue until the desired number of beads have been formed (see **Note 7**).
5. Allow a further 30 min of stirring before collecting the beads using a Büchner funnel.
6. Wash the beads with 10 times their volume of buffer appropriate for the enzyme. Remove the excess liquid. Storage of the beads is possible in the enzyme buffer until required (see **Note 8**).
7. Pour the wet alginate beads into the enzyme-maltose solution (see **Note 9**); the volumic ratio of enzyme-maltose solution/beads should be equal to 1 to reach a final activity of 200 U/mL in the reactor aqueous phase (see **Note 7**). This step should be performed at 4°C, and the mixture should be allowed to stand at this temperature for 24 h with occasional manual stirring.
8. Pack the tubular column up to half-full with the beads containing enzyme and maltose (**Fig. 1**) (see **Note 10**). Withdraw the excess liquid at the end of the operation (see **Note 11**).
9. Activate the heating device to allow the reactor to stand at the desired temperature for the reaction (40°C).

10. Circulate the water-saturated *n*-butanol mobile organic phase upward through the aqueous phase. The aqueous stationary phase volume should be constant, so that the alginate beads always stand in aqueous liquid (see **Notes** [12](#) and [13](#)).

11. Allow the upper part of the column to fill with the *n*-butanol phase, and collect the products (α -butylglucoside and glucose) at the outlet (top of the reactor) (see **Note** [14](#)).

4—

Notes

1. Calcium alginate beads are unstable in the presence of certain buffers and reagents that remove/complex the calcium ions and consequently destabilize the gel. Phosphate buffers and citrate buffers must be avoided, as well as chelating reagents, such as EDTA, and high concentrations of nongel-inducing ions, such as Na⁺ and Mg²⁺.

2. Pour the alginate powder slowly while the solution is continuously stirred; glycerol (up to 5%, w/w) might be added to assist the dissolving process. Stirring should be allowed to continue until the solution is homogeneous. Allow the solution to stand at room temperature for at least 1 h with occasional manual stirring (gently using a glass rod) to expel air bubbles.

3. α -Transglucosidases from other microorganisms can be used. For example, α -transglucosidase from *Talaromyces duponti*, which has been described as a thermostable enzyme (**18**), is appropriate for such purposes. However, in contrast to α -transglucosidase from *A. niger*, which is commercially available and ready to use, α -transglucosidase from *T. duponti* must be produced and partially purified to catalyze the stereospecific synthesis of α -butylglucoside (**5**).

4. The transglucosidase activity is assayed based on the liquid chromatography quantitative measurement of a transglucosylation product, panose, in an incubation mixture of enzyme and maltose (200 g/L) at 60°C and buffered in 50 mM sodium acetate, pH 5.0. The total volume is 1 mL. One unit of transglucosidase is defined as the amount of enzyme that produces 1 μ mol of panose/h at 60°C and pH 5.0.

5. The apparatus is suitable for small-scale production (volumes of sodium alginate up to 100 mL). For larger scale, use a peristaltic pump with tubing with a diameter around 1–2 mm to drop the pumped mixture at a rate of around 2.5 mL/min into the stirred calcium chloride solution.

6. Suitable average bead diameter is 2–3 mm. The number and size of the alginate beads are influenced by the size of the opening through which the alginate is allowed to pass. Short needles (not more than 5 mm to minimize the flow resistance in the needles) are advised.

7. One hundred grams of alginate solution give about 60 g of dry bead whose apparent volume is 80 mL (about 20 mL of interstitial volume if the average bead diameter is 3 mm).

8. Store the beads at 4°C for <1 wk. For longer storage (up to 3 mo), add maltose (200 g/L) in the buffer. Rinse with buffer appropriate for the enzyme before reuse.

9. During this step, the α -transglucosidase is loaded into the polymer. In fact, the high porosity of the alginate beads allows proteins to diffuse through the

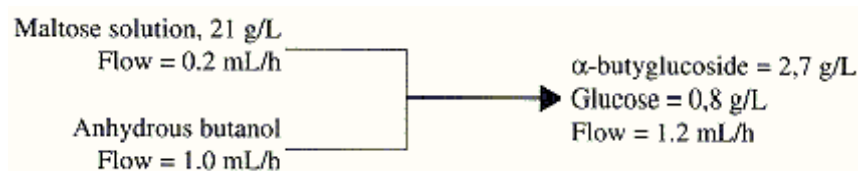
gel. Therefore, it will result in a partition of the enzyme between the beads and the solution.

10. The alginate beads are slowly added to the liquid. A gentle tamping (using a glass rod) is required during the operation to expel air bubbles. Air bubbles could prevent the butanol phase from distributing homogeneously in the reactor.

11. Care must be taken to ensure that the beads are always maintained in the aqueous liquid phase. Contact between the butanol phase and the beads would most likely result in inactivation of the enzyme, so a minimum aqueous liquid layer at the upper part of the stationary phase must be maintained.

12. The mobile phase on entry is composed of anhydrous butanol and of a maltose-containing aqueous phase. Flow rates are selected according to the working temperature: volumic ratios of organic and aqueous phase at the entrance of the reactor correspond to the water content of the water-saturated organic phase at the working temperature. For example, at 30°C, the aqueous flow should represent 13% (v/v) of the anhydrous butanol flow; at 40 and 50°C, it should be 16 and 20% (v/v), respectively. However, experimental adjustments are required for the reaction to perform correctly, in particular, for the stationary phase volume to stay constant and for the α -butylglucoside concentration, at the exit of the column, to be sufficient. For a 100-mL column and at 40°C, the total input flow should be about 10–20 mL/h.

13. Dry matter should be conserved, so the maltose concentration in the entering aqueous phase should be calculated from glucose and α -butylglucoside concentrations at the exit of the reactor. For example, in the conditions presented below, maltose concentration at the entrance of the column should be equal to 21 g/L:



α -Butylglucoside and glucose formation can be quantified by HPLC chromatography on an ion-exclusion column (PPH 224, Brownlee Labs, 4.6 mm internal diameter [id] $\times$ 220 mm). Elution conditions are: 5 mM sulfuric acid, flow rate: 0.3 mL/min, 25°C, injection volume: 10 μ L, detection: refractometer (RI-Detector 8110, ICS, France).

14. The partition coefficients of α -butylglucoside ($K_{\alpha BG}$) and glucose (K_{glucose}) (defined as the ratio of the α -butylglucoside or glucose concentration in the organic phase to the α -butylglucoside or glucose concentration in the aqueous phase) are equal to 1.05 and 0.025, respectively. The mixture is therefore composed of about 75% α -butylglucoside and 25% D-glucose. The glucose fraction at the outlet can be removed by liquid-liquid extraction with demineralized water. α -Butylglucoside, which is collected in the water-saturated *n*-butanol phase, is then concentrated by evaporation of the alcohol under reduced pressure and finally recovered at a purity above 95% (w/w). Do not maintain for a long time at temperatures higher than 90°C to avoid product degradation.

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24—

Enzymatic Glycosylation of Aglycones of Pharmacological Significance

Yukio Suzuki and Kei Uchida

1—

Introduction

There are numerous reports on the production of useful compounds by using biochemical reactions catalyzed by enzymes from microorganisms, plants, and animal organs. One of these areas of research is the biological and enzymatic transformation of useful compounds with stereospecific and regioselective modifications of chemical structures that are very difficult in synthetic organic chemistry. In plants most of the compounds that are unstable, toxic, and hydrophobic or volatile occur in glycosylated forms. These glycosylated compounds have excellent properties, such as a high stability, nontoxicity, and water-solubility. It follows that glycosylation is an excellent biotransformation method for preparing pharmacologically significant compounds with high stability, nontoxic, and water-solubility. We have worked on the enzymatic glycosylation of physiologically active compounds, such as vitamins (B₁, B₂, B₆, C), nucleosides (adenosine, inosine, uridine, arabinosylcytosine), sugars, sugar alcohols, flavonoids (rutin, hesperidin, naringin, naringin dihydrochalcone), aromatic, monoterpene, and indole alcohols, phenolic compounds, antioxidants, saponins, antibiotics, and so on by using glycosyltransferases (cyclomaltodextrin glucan-otransferase [CGTase] and dextranase) and various glycosidases from microorganisms and plants. The above glycosylated compounds have advantages over the original compounds (or aglycones) in their solubility in water, stability against ultraviolet light, heating, and air oxidation, reduction of a bitter taste and a stimulative tongue-pricking taste, and resistance to enzymatic action (1–3).

CGTase catalyzes three reactions, intramolecular transglycosylation, intermolecular transglycosylation, and hydrolysis, as follows:

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1. Intramolecular transglycosylation.

Starch $\rightarrow$ α -, β -, γ -cyclodextrins

2. Intermolecular transglycosylation.

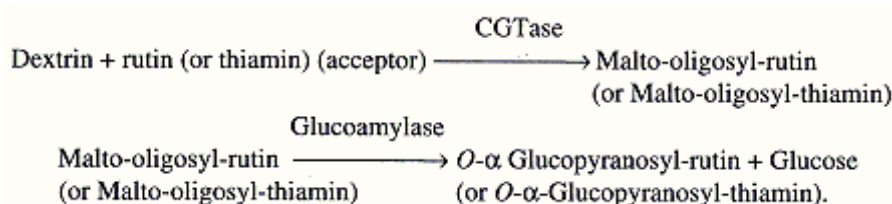
Starch + sugar (as acceptor) $\rightarrow$ Malto-oligosyl-sugar

3. Hydrolysis.

Starch (and cyclodextrin) $\rightarrow$ Malto-oligosaccharides

CGTases from *Bacillus macerans* and *B. megaterium* transglycosylate various monosaccharides by the 2nd reaction; the enzymes required a pyranose structure as an effective acceptor, which has the same configuration as glucopyranose, that is, with free C2, C3, and C4 hydroxy groups (4). Recently, CGTases from *B. stearothermophilus* and *B. circulans* have produced transfer products of sugar acceptors, such as galactose and mannose, which are very poor acceptors for the CGTases from *B. macerans* and *B. megaterium* (5). We found that *B. stearothermophilus* CGTase catalyzed the transfer reaction of the glucosyl residue from dextrin not only to OH group at C4 in glucose carbons of rutinose moiety in rutin (6) but also to CH₂OH groups of pyridoxine (7) and thiamin (8) with a high efficiency, and to OH group of phenolic compounds (3) at a considerable efficiency. *B. stearothermophilus* CGTase had much wider acceptor specificity than that previously reported (4).

Rutin occurs in many plants and is the most widespread of all quercetin glycosides. It has been suggested that rutin could be classed as a vitamin, particularly of the "Vitamin P" type. The transglycosylation to a water-insoluble flavonoid having a glucose residue, rutin, occurred readily in a water-methanol medium containing dextrin and rutin with *B. stearothermophilus* CGTase, and 4^G- α -glucopyranosyl-rutin was obtained in a high yield by the combined actions with glucoamylase from *Rhizopus* sp. (6). The solubility of α -glucosyl-rutin in water at 25°C was about 30×10^3 times higher than that of rutin. *O*- α -Glucopyranosylrutin is on the market today as a chemical reagent and added to foods and cosmetics.



Numerous derivatives of thiamin have been chemically synthesized. However, the glycoside of thiamin has been neither chemically nor biologically synthesized. In 1994, *O*- β -galactopyranosylthiamin (thiamin β -galactoside)

was first found by us to be formed from *O*-nitrophenyl- β -D-galactopyranoside and thiamin in 33% CH₃CN by *Aspergillus oryzae* β -galactosidase (9). More recently, we have obtained *O*- α -glucopyranosylthiamin (thiamin α -glucoside) from dextrin and thiamin by successive actions of *B. stearothermophilus* CGTase and *Rhizopus* sp. glucoamylase (8). This thiamin derivative is the second glycoside prepared of this vitamin. Both thiamin β -galactoside and thiamin α -glucoside were odorless and mildly sweet with no stimulative tongue-pricking taste. When 0.05 *M* aqueous solutions (adjusted to pH 7.0) of each of thiamin HCl, thiamin β -galactoside, and thiamin α -glucoside were allowed to stand overnight at room temperature, much odor was produced from the neutralized thiamin•HCl solution after 2 h, but both thiamin glycoside solutions were odorless even after 24 h. Thiamin β -galactoside and thiamin α -glucoside showed 50 and 73% activities of the equivalent moles of thiamin•HCl, respectively, when used in semi-synthetic diets by thiamin-deficient male rats. The biological effects examined were on the growth curve, food intake, liver weight, and the hepatic thiamin content. Thiamin glycosides, especially thiamin α -glucoside, may be useful derivatives of thiamin as food additives and medicines.

The present paper describes the enzymatic glycosylation of two compounds, rutin and thiamin, by *B. stearothermophilus* CGTase.

2—

Materials

2.1—

Enzymatic Formation of 4^G- α -Glucopyranosyl Rutin

1. For the assay of rutin and its derivatives, prepare the following reagents and instruments: Rutin (Merck); filter paper no. 50 (40 × 40 cm) for paper chromatography (Toyo Filter Paper Co., Tokyo, Japan); a solvent system of 1-butanolacetic-acid-water (2:1:1, v/v/v) (solvent A); a yellow fluorescence-producing reagent (The mixture of methanol and a mixed aqueous solution of AlCl₃ and CH₃COOK [1:1, v/v]). The mixed aqueous solution contains 8 g of AlCl₃ · 5H₂O and 49 g of CH₃COOK in 1 L of distilled water; ultraviolet (UV) lamp (3650Å filter); and a paper developing box.

2. For the assay of CGTase activity (10), prepare the following: soluble starch (Merck); 20 mM sodium acetate buffer (pH 5.5) containing 1 mM CaCl₂; 0.02 *N* H₂SO₄; and 0.1 *N* I₂ solution.

3. For paper chromatography (PPC) of rutin, its derivatives, and sugars, prepare: filter paper no. 50 (40 × 40 cm); solvent A; a solvent system of 1-butanol-pyridine-water (6:4:4, v/v/v) (solvent B); a yellow fluorescence-producing reagent; UV lamp (2537 Å and 3650Å filters); two paper-developing boxes; and sugar-detecting reagents comprising three solutions: Solution A is prepared by diluting 1 mL of saturated AgNO₃ to 6 mL with water and then to 200 mL with acetone. Solution B is the mixture of 10% aqueous NaOH and methanol (1:5, v/v). Solution C is 0.5 *M* Na₂S₂O₃ aqueous solution.

4. For enzymatic glycosylation reaction of rutin, prepare the following enzymes and reagents: α -glucosyl donor: dextrin (Pine-Dex #4, DE 18, Matsutani Chemical Mfa., Itami, Japan); acceptor: rutin; cyclomaltodextrin glucanotransferase (CGTase) from *B. stearothermophilus* (Hayashibara Biochemical Laboratories, Okayama, Japan); glucoamylase (grade I) from *Rhizopus* sp. (38.3U/mg) (Toyobo, Osaka, Japan); β -amylase from sweet potato (1,800 U/mg) (Boehringer Mannheim Japan, Tokyo, Japan); 0.2 M sodium acetate buffer (pH 5.5) containing 10 mM CaCl_2 ; methanol; a yellow fluorescence-producing reagent; solvent A; and filter paper no. 50 (40 $\times$ 40 cm).

5. Isolation of α -glucosylrutin: prepare the following enzymes and reagents: dextrin (Pine-Dex #4); rutin; CGTase from *B. stearothermophilus*; glucoamylase (grade I) from *Rhizopus* sp.; 1 M sodium acetate buffer (pH 5.5) containing 1.1 g CaCl_2 ; filter paper no. 50 (40 $\times$ 40 cm); Toyopearl HW-40S for column chromatography (Tosoh, Tokyo, Japan); methanol; solvent A; and phosphorus pentoxide.

6. For the characterization of α -glucosylrutin, prepare the following enzymes and reagents: α -glucosidase from rice (40–80 U/mg) (Sigma); α -glucosidase from yeast (50 U/mg) (Boehringer Mannheim); α -glucosidase from pig liver (1.3×10^3 U/mg) (**11**); α -glucosidase from *Mucor javanicus* (274 U/mg) (**12**); β -glucosidase from almond (2 U/mg) (Boehringer Mannheim); a solvent system of 1-butanol-acetic acid-water (4:1:5, v/v/v, upper layer) (solvent C); solvent A; quercetin (Merck); spectroscopic methanol (Nacalai Tesque); 1 N HCl; KBr for infrared absorption spectra measurements (Nacalai Tesque); and DMSO- d_6 (99.8%) (Euriso-top, CEA).

2.2—

Enzymatic Formation of α -Glucosylthiamin.

1. A high performance liquid chromatography (HPLC) analysis of thiamin and its derivatives: HPLC (Asahipack GS 320 column [7.6 $\times$ 500 mg] [Asahi Chemical Industries, Tokyo, Japan] is carried out using a Waters Associates chromatography system consisting of two pumps (model 6000A and 501G), an injector model U6K, a UV absorbance detector model 441, and a stainless steel mixing coil, together with a Shimadzu fluorescence HPLC monitor model RF 530, and two recorders (Shimadzu chromatopac model C-R1B). In addition, prepare the following reagents: thiamin hydrochloride (Nacalai Tesque); 0.04 M Sodium phosphate buffer, pH 6.0, and 0.01% $\text{K}_3(\text{Fe}[\text{CN}]_6)$ -15% NaOH solution.

2. For PPC of thiamin and its derivatives, prepare: filter paper no. 50 (40 $\times$ 40 cm); solvent A; and a blue fluorescence-producing reagent: A mixture of ethanol and an alkaline $\text{K}_3\text{Fe}[\text{CN}]_6$ solution (1:1, v/v). The alkaline solution contains 200 mL of 1% $\text{K}_3(\text{Fe}[\text{CN}]_6)$ aqueous solution, 26.4 mL of 30% NaOH and 13.6 mL of distilled water.

3. Enzymatic glycosylation reaction of thiamin: prepare the following enzymes and reagents: α -glucosyl donor: dextrin (Pine-Dex #1, DE 8); acceptor: thiamin hydrochloride; CGTase from *B. stearothermophilus* and its homogeneous preparation purified by the method of Kitahata and Okada (**13**); glucoamylase from *Rhizopus* sp. (grade I) (38.3 U/mg); a blue fluorescence-producing reagent; calcium chloride, anhydrous; and 0.1 M sodium acetate buffer, pH 5.5.

4. Isolation of α -glucosylthiamin: prepare the following enzymes and reagents: dextrin (Pine-Dex #1); thiamin hydrochloride; CGTase from *B. stearothermophilus*; glucoamylase from *Rhizopus* sp. (grade I); calcium chloride, anhydrous; 0.1 M sodium acetate buffer (pH 5.5); a blue fluorescence-producing reagent; filter paper no. 50 (40 × 40 cm); solvent A; activated Vitachange (Wako Pure Chemical Industries, Osaka, Japan); 25% (w/v) KCl aqueous solution; activated carbon; 85% ethanol; and phosphorus pentoxide.

5. For the characterization of α -glucosylthiamin, prepare the following enzymes and reagents: α -glucosidase from pig liver (see **Subheading 2.1.6.**); α -glucosidase from rice (see **Subheading 2.1.6.**); α -glucosidase from *M. javanicus* (see **Subheading 2.1.6.**); β -glucosidase from almond (20–40 U/mg) (Sigma); D₂O (Euriso-top, CEA); a solvent system of 1-propanol-2% NH₄OH (2:1,v/v); a solvent for HPLC: acetonitrile-water (9:1,v/v); kieselgel 600 plate for thin-layer chromatography (Merck); and Waters carbohydrate analysis column (4 × 300 mm).

3—

Methods

3.1—

Enzymatic Formation of 4^G- α -D-Glucopyranosyl-Rutin

3.1.1—

Assay of Rutin and its Derivatives

Rutin and its derivatives are assayed by Imai and Furuya's method (**14**) with a slight modification. A suitable amount of the reaction mixture containing rutin and its derivatives is applied as a band on filter paper no. 50 (40 × 40 cm) and developed twice by ascent in solvent A. After drying, the chromatogram is sprayed with a yellow fluorescence-producing reagent. Rutin and its derivatives on its chromatogram are shown as yellowish fluorescent bands under UV lamp (3650Å filter). Each yellow band is cut out and extracted with 20 mL of the mixed aqueous solution of AlCl₃ and CH₃COOK (1:1,v/v) at 25°C for 2 h. The amount of rutin compound in the extract is measured by the optical density at 420 nm.

3.1.2—

Assay of CGTase Activity (10)

Two hundred μ L of the enzyme solution is incubated with 5 mL of 0.3% soluble starch in 20 mM sodium acetate buffer, pH 5.5, containing 1 mM CaCl₂ at 40°C. After 10 min, 0.5 mL of the reaction mixture is pipetted out and added to 15 mL of 0.02 N H₂SO₄. To the mixture, 0.2 mL of 0.1 N I₂ solution is added, and then the color developed is measured at 660 nm. One unit of the enzyme activity is defined as the amount of the enzyme that catalyzes a 10% decrease of the absorbance per min under these conditions.

3.1.3—

Paper Chromatography

PPC is done on filter paper no. 50 with solvent A. After drying, the chromatogram is irradiated with UV lamp (2537Å filter) where the zones of rutin

and its derivatives are identified by their brownish yellow fluorescence. Also, rutin and its derivatives on the chromatogram are detected as yellow fluorescence under UV lamp (3650Å filter) after spraying with a yellow fluorescence-producing reagent. PPC of sugars is done with solvents A and B. The silver nitrate dip method (15) is used for sugar detection. The solutions (A, B, and C) are poured into three different Pyrex baking dishes, and the chromatogram is dipped into solution A and allowed to dry. The chromatogram is next dipped into solution B until the characteristic black spots of sugar appear. After washing with water, it is placed in solution C until the background coloration disappears. A final washing with water gives a stable chromatogram.

3.1.4—

Enzymatic Glycosylation Reaction of Rutin

CGTase (374 U) is incubated at 20°C for 48 h in the dark with a mixture (10 mL) of 0.5 g of dextrin in 5 mL of 0.2 M sodium acetate buffer, pH 5.5, with 10 mM CaCl₂ and 0.1 g of rutin in 5 mL of methanol (see Note 1). After incubation, many new yellowish-fluorescent spots with lower R_f values than that of rutin are detected besides rutin on a paper chromatogram (Fig.1). These spots are not formed in the reaction without dextrin and without CGTase. A 48-h incubation mixture is heated in a boiling water bath, and concentrated to remove methanol *in vacuo*. The concentrate is adjusted to pH 4.5 after dilution to 10 mL with water, and then incubated with glucoamylase (4.8 U) for 5 h at 50°C or with β -amylase (100 U) for 5 h at 37°C in the dark. As shown in Fig.1, much larger quantities of R-G₁ are formed when the 48-h incubation mixture with CGTase is digested with glucoamylase for 5 h. R-G₂ is formed as the main product after incubation with β -amylase. These results suggest that CGTase catalyzes the transfer of glucosyl residues from dextrin to rutin to form rutin malto-oligosaccharides.

3.1.5—

Isolation of α -Glucosylrutin

1. A reaction mixture (2 L), 100 g dextrin, 1.1 g CaCl₂, distilled water to make a volume of 760 mL, 20 g rutin in 1 L of methanol, 200 mL of 1 M sodium acetate buffer (pH 5.5), and 40 mL of *B. stearothermophilus* CGTase (74.8×10^3 U) are incubated at 20°C for 72 h in the dark under gentle stirring (73% of rutin as acceptor is transglycosylated to rutin glycosides) (see Note 2).
2. After incubation, the reaction mixture is heated in a boiling water bath and concentrated to about 100 mL. The concentrate, after dilution 5 times with distilled water, is adjusted to 4.5 with acetic acid, and then incubated with glucoamylase (958 U) at 50deg;C for 5 h in the dark (see Note 3).
3. The mixture, after pH adjustment to 6.0 and the addition of 1 vol of methanol, is heated for 10 min in a boiling water bath, and concentrated *in vacuo*.
4. The concentrate (400 mL) is put through the first preparative PPC with 400 sheets of filter paper no. 50 in solvent A, using 8 paper-developing boxes (see Note 4).

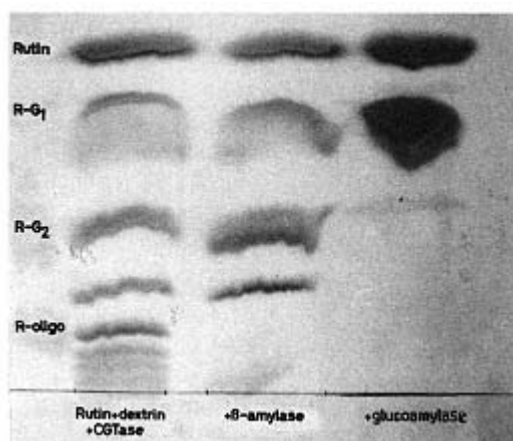


Fig. 1.

A paper chromatogram of reaction products formed from dextrin and rutin by successive actions of CGTase and either glucoamylase or β -amylase. 0.2 mL of each reaction mixture is applied on a paper and developed by ascent in solvent A. After drying the chromatogram is sprayed with a yellow fluorescence-producing reagent.

The band corresponding to R-G₁ is cut out, extracted overnight with 50% methanol at 37°C. The extract is concentrated, and put through the second PPC with the same solvent.

5. After appropriate sectioning, elution, and concentration, the concentrate is put on a Toyopeal HW-40S column (56 × 650 mm), washed with water, and eluted with 50% methanol. R-G₁ eluate, after concentration, is put on the second column chromatography using the same column (see **Note 5**).

6. R-G₁ eluate, after concentration and lyophilization, is further purified by treatment with methanol, water, and ethanol successively to remove insoluble materials and lyophilized. This purified powder of R-G₁ is crystallized from ethanol containing 0.001 N HCl. The needle crystals are dried *in vacuo* on P₂O₅ (232–235°C, decomp., $[\alpha]_{D16} + 65.3$ [$c = 3.77$, H₂O], FAB-MS data: $[M + H]^+$ ion at 773) (yield 4.07 g). 2.15 g of R-G₁ dissolves in 0.5 mL of water at 25°C, while 1 g of rutin dissolves in 8 L of water. This shows that enzymatic transglycosylation can be used for the improvement of solubility of insoluble compounds in water.

3.1.6—

Characterization of α -Glucosylrutin

1. R_f values (0.17, 0.45) of the isolated R-G₁ on PPC in 2 solvent systems (solvents A and C) are different from those of rutin (0.50, 0.64) and quercetin (0.69, 0.74).

2. On acid hydrolysis with 1 N HCl at 100°C for 1 h in the dark, the products are sugars and a yellow substance, which is isolated in crystalline form and identified as quercetin by R_f values, mp, UV, and infrared absorption spectra in comparison with those of the authentic specimen. The sugar components in the hydrolyzate are confirmed as glucose and rhamnose by PPC and HPLC.

3. R-G₁ is readily hydrolyzed by α -glucosidases from rice, *M. javanicus* and pig liver, and weakly by yeast α -glucosidase, but not by almond β -glucosidase. The enzymatic hydrolysis of R-G₁ releases rutin and glucose (molar ratio, 1:1), which are identified by PPC and HPLC, respectively (see **Note 6**).

4. Six UV absorption spectra comprising one in methanol and five obtained by adding diagnostic reagents to R-G₁ in methanol show changes of the position of maxima and minima closely similar to those of rutin (see **Note 7**). The IR absorption of R-G₁ (KBr pellet) resembles that of rutin.

5. The examination of ¹H-NMR spectrum (in DMSO-*d*₆) of R-G₁ shows a rhamnosyl methyl signal (0.97, 3H, d), signals of ring protons of the rhamnomaltosyl group (3.00–3.80 ppm, 16 H), 3 anomeric proton signals of the rhamnosidic (4.45, 1H, s), α -glucosidic (4.98, 1H, d, *J* = 3.9 Hz) and β -glucosidic linkages (5.34, 1H, d, *J* = 7.7 Hz), and 5 methyne signals of H-6 (6.12, 1H, d, *J* = 2.0 Hz), H-8 (6.30, 1H, d, *J* = 2.0 Hz), H-5' (6.84, 1H, d, *J* = 8.4 Hz), H-2' (7.52, 1H, d, *J* = 2.2 Hz), and H-6' (7.55, 1H, q, *J* = 10.5 Hz). There are negligible differences for 5 methyne signals between R-G₁ and rutin.

6. Carbon-13 chemical shifts of R-G₁ in DMSO-*d*₆ are compared with those of rutin (**Table 1**). Glucosylation of rutin causes a large chemical shift of C-4 with a concomitant small shift of C-5 in glucose carbons, and considerably smaller effects on rhamnose carbons in a rutinose moiety. The chemical shifts of two glucose carbons of R-G₁ are similar to those of maltotriose (16). There are negligible differences for carbons in the quercetin moiety between rutin and R-G₁.

7. From these results, it is apparent that the glucosylation site is present at C-4 in glucose carbons of the rutinose moiety. R-G₁ is identified as 4^G- α -D-glucopyranosyl-rutin (**Fig. 2**).

3.2—

Enzymatic Formation of α -Glucosylthiamin.

3.2.1—

Assay of Thiamin and its Derivatives

Thiamin and its derivatives are analyzed by the method of Kimura et al. (17) with slight modification, using HPLC. The mobile phase (0.04 M sodium phosphate buffer, pH 6.0), is pumped in a tube (inside diameter, 0.23 mm) at a flow rate of 1 mL/min by a Waters Associates chromatography pump model 6000A. A sample of a solution containing thiamin and its derivatives is injected by a Waters injector model U6K on to the column (Asahipack GS-320). Thiamin and its derivatives in the effluent are first measured at a wavelength of 280 nm with a Waters UV absorbance detector, model 441, connected to a Shimadzu chromatopac model C-R1B. Then a 0.01% K₃(Fe[CN]₆)-15% NaOH solution is put in at 1 mL/min by a Waters 501 G pump, and mixed in a stainless steel mixing coil (0.51 × 1500 mm) with the column effluent to convert thiamin compounds into fluorescent compounds. The fluorescent compounds are measured with a Shimadzu fluorescence HPLC monitor model RF 530 (excitation 375 nm, emission 435 nm), connected to a Shimadzu chromatopac model C-R1B and recorded graphically. The peak area method is used for measurement.

Table 1
¹³C-NMR Spectra of Sugar Carbons of Both Rutin and R-G₁

Carbon number	Rutin (ppm)	R-G ₁ (ppm)	Difference ^a (ppm)
Rutinoses carbon			
Glucose carbon			
1	100.5	100.6	-0.1
2	73.8	73.4	0.4
3	76.2	75.9	0.3
4	70.1	79.8	-9.7
5	75.7	73.7	2.0
6	66.8	66.5	0.3
Rhamnose carbon			
1	100.9	101.1	-0.2
2	70.3	70.3	0.0
3	69.8	69.3	0.5
4	71.6	71.6	0.0
5	68.2	68.1	0.1
6	17.5	17.5	0.0
Transferred glucose carbon			
1'		101.2	
2'		73.0	
3'		73.3	
4'		69.8	
5'		72.2	
6'		60.3	

^aThe figures represent the difference of chemical shift between rutin and R-G₁.

3.2.2— Paper Chromatography

PPC is done in a filter paper no. 50 (40 × 40 cm) by 2 ascending developments with solvent A. After drying, thiamin and its derivatives on chromatograms are detected as bluish-fluorescent bands under UV lamp (3650Å filter) by spraying with a blue fluorescence-producing reagent.

3.2.3— Enzymatic Glycosylation Reaction of Thiamin

A reaction mixture (10 mL, adjusted to pH 5.5) containing 0.5, 1.0, or 1.5 g of dextrin, 0.5 g of thiamin in the center like thiamin · HCl, 5 mL of 0.1 *M* sodium acetate buffer, pH 5.5, 5.5 mg of CaCl₂, 680 U of a purified preparation of CGTase, and distilled water to make a total volume of 10 mL, is incubated at 37°C for 72 h in the dark. After incubation, many new thiochrome reaction-positive spots with lower *R_f* values than that of thiamin are detected besides

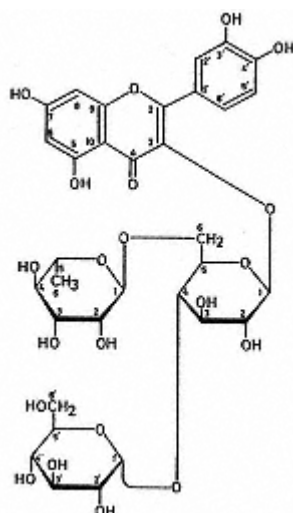


Fig. 2.
Structure of R-G₁, 4^G-α-D-
glucopyranosyl-rutin.

thiamin on a paper chromatogram. These spots are not formed in the reaction mixture without dextrin and without enzyme. The formation of these new spots from thiamin with 15% dextrin is higher than those with 5 or 10% dextrin. Furthermore, two thiochrome reaction-positive (major and very minor compounds [I and II]) having lower R_f values than that of thiamin on a paper chromatogram are formed when the 72-h incubated reaction mixture is digested with 30 U of glucoamylase at 37°C for 20 h (**Fig. 3**). We suppose that these new spots may be transglycosylated derivatives of thiamin.

3.2.4—

Isolation of α-Glucosylthiamin

1. The reaction is run on a large scale with 75 g dextrin, 25 g thiamin in the center like thiamin · HCl, 250 mL of 0.1 M sodium acetate buffer, pH 5.5, 275 mg CaCl₂, 34 × 10³ U CGTase, and distilled water to make a total volume of 500 mL (pH adjusted to 5.5) at 37°C for 72 h in the dark.
2. After the pH is adjusted to 4.5, the reaction mixture is heated in a boiling water bath for 10 min in the dark to stop the enzymatic action and centrifuged. The supernatant solution is incubated with 1500 U of glucoamylase at 37°C for 20 h in the dark.
3. After being heated in a boiling water bath for 10 min, the mixture is applied to the first preparative PPC with 500 sheets of filter paper no. 50 (40 × 40 cm) in solvent A, using 8 paper-developing boxes. Two bluish-fluorescent bands corresponding to major and very minor compounds (I and II) in addition to that of thiamin are observed on the paper chromatograms, after spraying the mixture of ethanol and an alkaline K₃(Fe[CN]₆) solution (1:1,v/v). The band position corre-

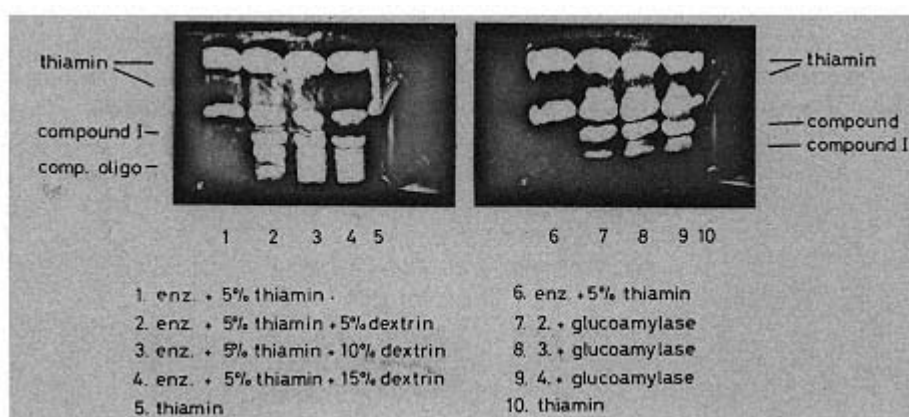


Fig. 3.

Paper chromatograms of reaction products formed from dextrin and thiamin by successive actions of CGTase and glucoamylase. Compound I, thiamin α -glucoside; Compound II, thiamin α -maltoside-like compound; Comp. oligo, thiamin α -malto-oligosaccharides-like compound.

sponding to the major one (compound I) on all paper chromatograms is cut out and extracted with acetic acid aqueous solution, pH 4.2, (see **Note 8**). The amount of compound I in the extract is analyzed by HPLC (7.50 gas thiamin $\cdot$ HCl). The extract is concentrated below 30°C *in vacuo*, and then lyophilized.

4. The preparation of compound I dissolved in water is applied to an activated Vitachange column (5.6 $\times$ 16 cm) at 4°C. After the column is washed completely with water to remove sugars, compound I is eluted with a 25% KCl aqueous solution at 4°C. To the eluates containing compound I and contaminants (i.e., thiamin, compound II, and a large amount of KCl), ethanol is added to a final concentration of 75% at 0°C. The resultant precipitate is removed by filtration. The filtrate is concentrated and lyophilized.

5. The powdered preparation is extracted three times with 85% ethanol. The alcoholic extract, after concentration, is subjected to the 2nd PPC with solvent A, to completely separate compound I from contaminants (thiamin and compound II). After appropriate sectioning and elution, the compound I solution is concentrated and lyophilized (see **Note 9**).

6. The preparation, after dissolved in 85% ethanol, is decolorized with activated carbon, concentrated, and then lyophilized. A small amount of methanol is added to the powdered preparation, and the insoluble materials are removed. The filtrate is concentrated and lyophilized. The purified powdered preparation of compound I is crystallized several times from 1-butanol-methanol solution (see **Note 10**). The crystalline preparation is dried *in vacuo* on P₂O₅ (yield, 2.38 g). The melting point and FAB-MS data are as follows: mp (decomp.): 184–186°C; [M+H]⁺ ion at 427 (thiamin: [M+H]⁺ ion at 265). This crystalline preparation is odorless and mildly sweet with no stimulative tongue-pricking taste.

3.2.5—

Characterization of α -Glucosylthiamin

1. On enzymatic hydrolysis with α -glucosidases from pig liver, rice, and *M. javanicus*, compound I is hydrolyzed completely to a sugar and a thiochrome reaction-positive substance, which has R_f values identical to those of thiamin on paper chromatograms. The sugar component in the hydrolyzate is confirmed as glucose on a Kieselgel 60 plate developed with a solvent system of 1-propanol-2% NH_4OH (2:1,v/v) and HPLC using a Waters carbohydrate analysis column developed with CH_3CN -water (9:1,v/v). The molar ratio of thiamin and glucose liberated is around 1:1 in compound I. No hydrolysis occurs with almond β -glucosidase.

2. The UV_{max} value of the compound I is 248 nm in 0.1 N HCl, 235 and 268 nm in 0.1 M sodium phosphate buffer (pH 6.8), and 232 and 338 nm in 0.1 N NaOH solution. These values are very similar to those of thiamin.

3. Examination of the ^1H -NMR spectrum of compound I (in D_2O , ppm) shows the following signals: 2.57 (thiazole- CH_3 , H-9, s, 3H), 2.64 (pyrimidine- CH_3 , H-12, s, 3H), 3.34 (inner- CH_2 - of side chain, H-10, t, 2H), 3.82 ($-\text{O}-\text{CH}_2\text{H}_b$ -side chain, H-11_b, m, 1H), 4.00 ($-\text{O}-\text{CH}_2\text{H}_a$ -side chain, H-11_a, m, 1H), 5.58 (bridge- CH_2 -, H-5, s, 2H), and 8.04 (aromatic proton in pyrimidine ring, H-2, s, 1H). These signals are assigned to the protons of thiamin based upon the data reported (18). Additional signals are as follows: signals of ring protons of the sugar moiety (3.36–3.81 ppm, 6H) and one anomeric proton signal of an α -glucosidic linkage (4.97, 1H, d, $J = 4.0$ Hz).

4. Carbon-13 chemical shifts of compound I are compared with those of thiamin (Table 2). Signal assignments are based on the data reported for methyl- α -glucopyranoside (19) and thiamin (20) in D_2O . It is apparent that glucosylation site in compound I is the CH_2OH group in the 4-methyl-5-hydroxyethyl thiazolium moiety, since the appended C-11 signal is downfield shifted by a sizable 5.70 ppm with a concomitant small upfield shift of C-10 in the hydroxy group and only smaller effects on the thiamin carbons.

5. Thus, compound I is identified as 5'-O-(α -D-glucopyranosyl) thiamin (Fig. 4).

4—

Notes

1. In a transglycosylation reaction, a high concentration of acceptor in a reaction mixture is generally desirable. The solubility of rutin in water is very low, but very high in methanol. However, enzymes become unstable in water-organic solvent mixtures and lose their activity. In preliminary experiments, several CGTases were shown to maintain sufficient ability for the intermolecular transglycosylation reaction to occur in 50% aqueous methanol solution for 3 d at 20°C, but not 37°C. The solubility of rutin in 50% aqueous methanol solution is about 10 times higher than that in water. The CGTases from *B. stearoothermophilus* and *B. circulans* showed a higher transglycosylation activity to rutin, but the two CGTase preparations from *B. macerans* had very weak activity.

2. The reaction mixture is prepared in the following way: 20 g of rutin are dissolved in 1 L of methanol; to the methanol solution of rutin is added the dextrin aqueous solution containing 100 g dextrin and 1.1 g CaCl_2 in 760 mL distilled water; if

Table 2
¹³C-NMR Chemical Shifts of Thiamin and Compound I in D₂O

Carbon no.	Thiamin (ppm)	Compound I (ppm)	Difference (ppm)
Thiamin carbon			
C-1	164.13	163.89	0.24
C-2	145.72	145.57	0.15
C-3	107.21	106.95	0.26
C-4	164.38	164.19	0.19
C-5	51.00	50.74	0.26
C-6	155.72	155.69	0.03
C-7	143.95	143.72	0.23
C-8	137.67	137.16	0.51
C-9	12.10	11.90	0.20
C-10	30.30	27.59	2.71
C-11	61.33	67.03	-5.70
C-12	22.04	21.85	0.19
Transferred glucose carbon			
C-1		99.18	
C-2		71.89	
C-3		73.88	
C-4		70.40	
C-5		73.21	
C-6		61.46	

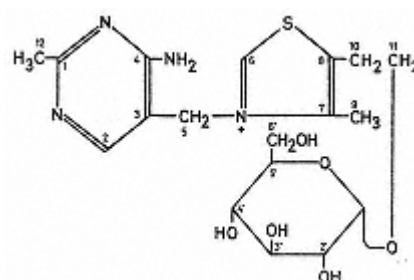


Fig. 4.
 Structure of compound I, 5'-O-(α-D-glucopyranosyl) thiamin.

any precipitate of rutin or turbidity appears, the mixed solution is heated in a boiling water bath without the addition of 1 M acetate buffer, pH 5.5, in order to avoid the deep coloration of the mixed solution; after cooling to 20°C, 200 mL of 1 M acetate buffer, pH 5.5, and 40 mL of CGTase are added.

3. Glucoamylase produces a large quantity of R-G₁ by consecutively removing the glucose units from the nonreducing end of rutin malto-oligosaccharides, and also hydrolyzes most of the residual dextrin and malto-oligosaccharides formed in a 72 h incubation mixture with CGTase only into glucose. The viscosity of the 72-h incubation mixture is decreased. In a prolonged incubation at 50°C with a large amount of glucoamylase, rutin malto-oligosaccharides are hydrolyzed to rutin and glucose.
4. The concentrate is applied as bands (1 mL aliquot of the concentrate per a sheet of filter paper, 40 × 40 cm) along a starting line previously drawn in parallel to the bottom of the sheet. Sixty-four sheets of filter paper are developed for 36 h by ascent in solvent A in 8 paper-developing boxes. R-G₁, having intermediate R_f value between rutin and glucose on chromatogram is effectively separated from large amounts of both rutin and glucose by PPC.
5. The column with a Diaion HP 10 (Mitsubishi Chemical Mfa, Tokyo, Japan) can be used to separate both rutin and R-G₁ from sugars. Rutin and R-G₁ in the concentrate are adsorbed on the column, washed with water to remove sugars, and eluted with 50% methanol. Then gel-filtration on Toyopearl HW 40S column is used to separate R-G₁ from rutin.
6. The hydrolysis of R-G₁ by α - and β -glucosidases are examined as follows. The reaction mixture containing 4 mg of R-G₁, 5 U of enzyme, and buffer in a total volume of 1 mL is incubated for 20 h. Control experiments are done out with 2 mg of rutin. 0.1 M sodium acetate buffer (pH 5.0) is used in the reaction with *M. javanicus* α -glucosidase at 50°C, 0, 1 M sodium acetate buffer (pH 5.0) with rice α -glucosidase at 37°C, 0.1 M sodium phosphate buffer, pH 7.0, with yeast α -glucosidase at 37°C, 0.1 M sodium phosphate buffer, pH 5.0, with pig liver α -glucosidase at 50°C, and 0.1 M sodium acetate buffer, pH 5.0, with almond β -glucosidase at 37°C. The reaction is stopped by heating for 3 min in a boiling water bath. Rutin and sugar liberated during the reaction are examined by PPC and HPLC. The amounts of rutin and sugar (glucose) released are estimated by the modified method of Imai-Furuya (14) and the method of Nelson (21), respectively.
7. UV absorption spectra are measured in six kinds of solvents (22): methanol (MeOH); MeOH + NaOH; MeOH + AlCl₃; MeOH + AlCl₃ + HCl; MeOH + sodium acetate (NaOAc); and MeOH + NaOAc + boric acid.
8. Each band position corresponding to compounds I and II on all paper chromatograms is cut out, after all strips at both edges of the chromatogram are made visible using a blue fluorescence producing reagent.
9. In a small-scale preparation of compound I, the 7-hr incubation mixture digested with glucoamylase, after five times dilution with water, is directly applied to an activated Vitachange column without the preparative PPC and washed with water to remove sugars. Then, thiamin and compound I are eluted with 25% KCl aqueous solution. Compound I is separated from thiamin by gel filtration on Toyopearl HW-40S column.
10. 1-Butanol is added slowly, dropwise to the methanol solution of compound I until turbidity appears.

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25—

Enzymatic Synthesis of Glycosides in Aqueous-Organic Two-Phase Systems and Supersaturated Substrate Solutions

Jeffrey A. Khan, Anna Millqvist-Fureby, and Evgeny N. Vulfson

1—

Introduction.

In order to prepare anomerically pure glycosides by traditional chemical methods, it is either necessary to perform a conventional protection/activation/coupling/deprotection sequence or to synthesize the glycoside as a mixture of anomers which require subsequent resolution (**1,19**). Enzymes have a dual attraction as catalysts for this type of reaction. First, a wide range of very inexpensive glycosidases are available commercially; second, they provide absolute stereochemical control at the anomeric center of the newly synthesized glycosydic bond. Consequently, the synthetic potential of glycosidases has been actively explored over the last decade with considerable research focusing on the use of enzymes for the preparation of oligosaccharides. Keen interest in the development of enzymatic methods for the glycosylation of sugars arose from drawbacks associated with conventional coupling chemistry and the requirement for regioselective protection of hydroxyl groups. Despite notable successes (*see* other chapters in this book), the control of regioselectivity in glycosidase-catalyzed reactions remains the major challenge in this area (**2**), with the efficiency of glycosylation arguably being a secondary issue.

It is also attractive to utilize enzymes for the glycosylation of hydrophobic substrates to include targets ranging from relatively simple medium/long-chain alkylglycosides to more complex bioactive compounds such as steroids. In this case, however, the efficiency of the reaction becomes a critical factor due to the low solubility of these alcohols in water that are routinely used as a medium for enzymatic transformations of oligosaccharides. Attempts to perform the

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transformation of hydrophobic substrates in nearly anhydrous organic solvents rather than aqueous buffers have been largely unsuccessful so far because glycosidases, unlike other hydrolytic enzymes, do not seem to function particularly well in very low water environments, even after immobilization (3) or chemical derivatization (4). In some instances, this problem has been overcome by using appropriate water miscible cosolvents to keep both reactants, i.e., the sugar and alcohol, in solution. Indeed, there are examples in the literature when enzymatic glycosylations have been carried out in a medium containing up to 50% acetone, acetonitrile, *tert*-butanol, and dimethyl ethers of mono-, di-, and triethylene glycol (5–10). Unfortunately, most enzymes are too unstable under these conditions and undergo rapid and irreversible denaturation in the presence of intermediate concentrations of water-miscible cosolvents (11). However, when the co-solvent content in the mixture is further increased to about 90%, glycosidase stability does improve dramatically (12–14). Several laboratories have recently described the synthesis of alkylglycosides in predominantly organic media, although in most cases the yields were not very high (12–16).

Aqueous-organic two-phase systems offer a good alternative for enzyme stabilization. However, a common drawback of two-phase systems is that of the relatively low-reaction rates exhibited by the enzymes. This is due to the “compartmentalization” of the water-soluble and hydrophobic substrate in the aqueous and organic phase, respectively, with the enzyme contained in the aqueous phase. There is little advantage in using glycosides or disaccharides under these conditions because they will be rapidly hydrolyzed by the enzyme in the aqueous phase. Therefore, the reaction pathway is usually the reversal of hydrolysis rather than transglycosylation (i.e., the transfer of the glycon from an activated substrate to the accepting alcohol). Hence the reaction takes longer to reach equilibrium due to two factors: the slower rate of formation of the enzyme-bound oxonium intermediate from monosaccharides (Fig. 1) and a relatively low steady state concentration of the hydrophobic acceptor (alcohol) in the aqueous phase leading to mass transfer limitations. Nonetheless, aqueous-organic two-phase systems have been successfully employed by several authors for the preparation of medium chain alkylglycosides (3,17–23). The main objective of this paper is to describe both the traditional method of enzymatic glycosylation and some novel approaches which were designed to improve the efficiency of reactions involving poorly water-soluble alcohols.

Clearly the reaction rate, and hence the overall productivity, in an aqueous-organic two-phase system should improve if the aqueous phase is dispersed in the solvent in the form of small droplets stabilized, at least for the time necessary for the biotransformation. In principle this should be possible by using reverse micelles containing the enzyme and a concentrated solution of

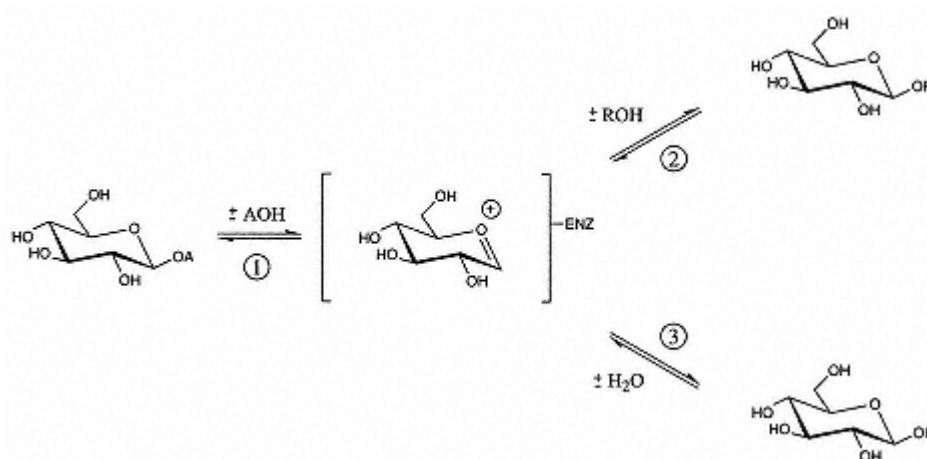


Fig. 1.

The mechanism of enzymatic (trans)glycosylation. AOH (e.g., p-nitrophenol) and ROH (e.g., a primary alcohol) are glycon donor and acceptor respectively; the oxonium ion in brackets is an enzyme bound intermediate.

monosaccharide with the alcohol included in the organic phase. Furthermore, if the resulting alkylglycoside can be used to stabilize the micelles, the problem of removing another surfactant from the reaction mixture will be eliminated. Unfortunately, virtually no product was formed under the experimental conditions used by Legoy and coworkers (24), by whom this elegant approach was attempted.

Alternatively, the aqueous droplets can be stabilized in the form of permeable, polymeric microcapsules prepared by interfacial polymerization under mild conditions to minimize biocatalyst inactivation. This procedure was originally developed in our laboratory for the immobilization of microorganisms (25) and, more recently has been applied to the microencapsulation of β -glucosidase (26). As above, the synthesis was carried out in a two phase reaction mixture: polymeric microcapsules, containing enzyme and a saturated solution of glucose (aqueous phase), were suspended in primary alcohol that formed the organic phase. Various other transglycosylation reactions should have taken place under these conditions, but the resulting oligosaccharide products were confined to the aqueous interior of the capsule while the relatively hydrophobic alkylglucoside was extracted into the solvent where it accumulated in a practically pure form (Fig. 2). The major advantages of this methodology are improved productivity due to enhanced mass transfer and the possibility of performing the synthesis continuously in a specially designed bioreactor (26).

Since glycosylation in any aqueous-organic two-phase system is essentially a thermodynamically controlled process, the reaction yields depend on the

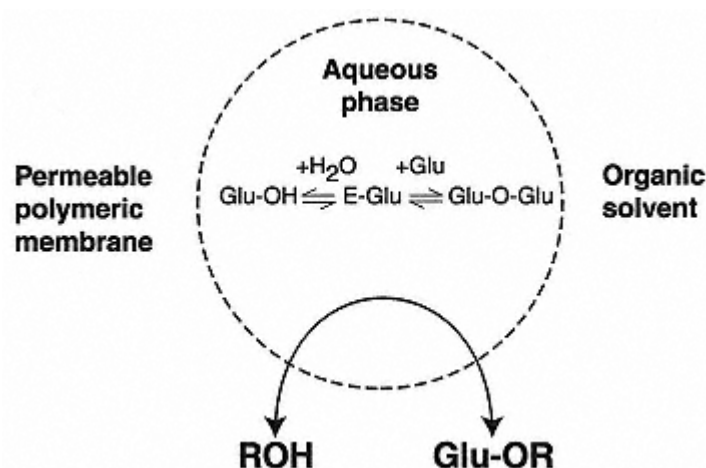


Fig. 2.

A schematic representation of enzyme-catalysed synthesis of alkyl- β -[D]-glucopyranosides using microencapsulated β -glucosidase.

volume ratio between the phases, the initial concentration of the reagents in the system, and the partition coefficient of the substrates and products (see **ref. 21** for an excellent thermodynamic analysis). The latter parameter is also important for the kinetics of the reaction. As shown in **Table 1**, conventional and microcapsule-based systems gave very similar yields of hexyl- and octyl- β -glucoside when the reaction was carried out under the same conditions, although thermodynamic equilibrium was reached noticeable faster with the encapsulated biocatalyst. However, virtually no product was observed in both systems with relatively long-chain alcohols even after prolonged incubation periods, despite the expected favorable thermodynamics, because of the negligible solubility of the accepting alcohol in water. Lower yields were also observed with relatively hydrophilic acceptors such as α,ω -diols, presumably due to the less favourable thermodynamics. In order to increase yields in this and similar reactions, we have recently developed an alternative approach which is suitable for the glycosylation of poorly water-soluble, but not too hydrophobic, substrates.

It has been shown that enzymatic (trans) glycosylation can be carried out efficiently in supersaturated (supercooled) solutions of substrates, consisting of monosaccharide/glycoside and the accepting mono/diols, using a range of crude commercially available glycosidases (**27**). These solutions, formed in the presence of small amounts of water and an alcohol-acceptor used as plastisizers, were sufficiently stable under ambient conditions to enable enzymatic transformations to occur. In particular, monosaccharides such as glucose readily formed metastable supersaturated solutions with α,ω -diols containing

Table 1
Comparison of Enzymatic Glucosylation in a Conventional Aqueous-Organic Two-Phase System and in Microcapsules^a

Reaction time, <i>h</i>	Concentration of hexyl-β-[D]-glucopyranoside, mM		Concentration of octyl-β-[D]-glucopyranoside, mM	
	XAD-4	Microcapsule	XAD-4	Microcapsule
5	12	26	4	14
24	32	40	9	19
72	40	40	18	22

^aAll reactions were performed at a fixed enzyme loading (560U) at 50°C in *n*-hexanol (13.5 mL) and buffer, pH 5.6 (1.5 mL).

Table 2
β-Glucosidase Catalyzed Synthesis of α,ω-Hydroxyalkyl-β-[D]-Glycosides in Supersaturated Solutions

Substrate	α,ω-Diol	Diol:β-glucoside (mole:mole)	Reaction time, <i>h</i>	Yield, %
Glucose	1,6-hexanediol	1.5:1	72	41
Ehtyl-β- <i>O</i> -glucoside ^a	1,6-hexanediol	3.3:1	2 ^a	43 ^b
Ehtyl-β- <i>O</i> -glucoside ^a	1,8-octanediol	2.6:1	4.3	36 ^b
Butyl-β- <i>O</i> -glucoside ^a	1,6-hexanediol	5.5:1	2	42 ^b
Butyl-β- <i>O</i> -glucoside ^a	1,8-octanediol	4.3:1	6	33 ^b

^aAnomeric mixtures of the alkyl-[D]-glucosides were prepared using the procedure of Theil et. al. (30) and used as obtained for all reactions.

^bYields were calculated on the basis of conversion of the β-anomer of the substrate.

up to six carbon atoms, and further chain-length extension was possible using short-chain alkylglucosides (**Table 2**). In the latter case, although the reaction was kinetically controlled, hydrolysis of the glycoside was noticeably reduced due to the relatively low-water content of the medium. The practical utility of this new approach was demonstrated by the preparation of several new emulsifiers and the regioselective synthesis of disaccharides (28,29). Typical examples of biotransformations carried out in aqueous-organic two phase systems and supersaturated solutions together with relevant analytical methods are described in detail in **Subheadings 2 and 3**.

2—

Materials

β-Glucosidase (EC# 3.2.1.21) from almonds, 2-(*N*-morpholino)-ethanesulfonic (MES), dithiothreitol (DTT), bis(trimethylsilyl)trifluoroacetamide (BSTFA),

XAD-4, orcinol-FeCl₃ spray reagent, and citric acid were obtained from Sigma (Dorset, UK). α -[D]-Glucose, low molecular weight poly(allylamine hydrochloride), 1,6-hexanediamine, 1,6 hexanol, 1,8 octanol, dodecanedioyl chloride, sorbitan trioleate (Span 85) and all organic solvents were supplied by Aldrich Chemical (Dorset, UK). The organic solvents used in this study were of the highest purity available and were routinely stored over 4Å molecular sieves (Aldrich).

3—

Methods

3.1—

Immobilization of β -Glucosidase on XAD-4 (17)

1. Prior to immobilization, XAD-4 is extensively rinsed with methanol, 10 mM acetate buffer pH 5.0, and distilled water. The excess of water is removed by filtration and the support dried.
2. Almond β -glucosidase is dissolved in 10 mM acetate buffer, pH 5.0, at a concentration of 20 mg/mL and 1 g of dry support is added per 1 mL of the enzyme solution.
3. After mixing, the preparation is dried under a stream of nitrogen and stored in a vacuum desiccator over P₂O₅. All preparations are made at room temperature.

3.2—

Synthesis of Hexyl- and Octyl- β -[D]-Glucopyranoside in a Conventional Aqueous-Organic Two-Phase System (18)

1. In a typical experiment, 0.75 g of XAD-4 immobilized enzyme is added to the reaction mixture consisting of 20 mL primary alcohol and 1.6 mL (for hexanol) or 1.0 mL (for octanol) of the acetate buffer containing 2.5 M glucose. The reaction is performed at 60°C in 50-mL Duran flasks in a Luckham R300 shaker-incubator at 150 rpm. The progress of the reaction is followed by high-pressure liquid chromatography (HPLC) as described below. Under these conditions, 38 mM of hexyl- β -[D]-glucopyranoside and 22 mM of octyl- β -[D]-glucopyranoside are formed after 3 and 5 d of incubation, respectively.
2. After the reaction is completed and the biocatalyst removed by filtration, the alcohol phase is washed twice with distilled water to remove traces of glucose, and the solvent is removed by rotary evaporation to give ~120–150 mg of product, depending on the alcohol used.

3.3—

Preparation of Polymeric Microcapsules Containing β -Glucosidase (25,26)

1. β -Glucosidase (80 mg, 560 U) and α -[D]-glucose (1600 mg) are dissolved in 0.75 M MES buffer, pH 6.6 (1.5 mL) containing 0.2 M poly(allylamine hydrochloride), with stirring for 15 min at 50°C.
2. This solution is then added dropwise to 15 mL decane containing 220 μ L Span 85 with stirring using an IKA overhead blender and maintenance of the reaction temperature at 50°C.
3. To the resulting suspension, 45 μ L dodecanedioyl chloride in 10 mL dry decane is added dropwise over 10 min and the mixture is stirred for a further 5 min.

4. Stirring is then continued at room temperature, using a conventional magnetic stirrer, for a further 30 min.

5. The solvent is decanted from the microcapsules (30–50 μm diameter), which are then rinsed with decane ($2 \times 10 \text{ mL}$) and water-saturated alcohol ($2 \times 10 \text{ mL}$), decanted, and used for the biotransformation.

3.4—

Synthesis of Hexyl- and Octyl- β -[D]-Glucopyranoside Using Microencapsulated Biocatalyst (26)

1. A batch of microcapsules (1.5 mL aqueous volume) containing 80 mg of β -glucosidase (560 U) are added to water saturated *n*-hexanol or *n*-octanol (13.5 mL) and sealed in 50-mL Duran bottles. The reaction is carried out at 50°C in a Luckham R300 shaker-incubator at 150 rpm.

2. The conversion is followed by withdrawing 0.25 mL aliquots of the reaction mixture. These samples are diluted with 0.75 mL hexanol and analyzed by HPLC.

3. After 24 h incubation, when the concentration of hexyl- and octyl- β -[D]-glucopyranoside in the organic phase reaches 40 and 19 mM respectively, the biocatalyst is filtered off and the product recovered as described above.

3.5—

HPLC Analysis

1. This was performed using Gilson 305/306 pumps (Anachem, Luton, UK) equipped with a Sedex 55 (Sedere, Paris, France) evaporative light scattering detector (evaporation temperature = 45°C; nitrogen pressure 1.9 bar) and a Spherisorb ODS2 $250 \times 4.6 \text{ mm}$ column (Hichrom, Reading, UK) eluted with a linear gradient of acetonitrile/water (from 20 to 70% of acetonitrile) which is applied over 7 min at a flow rate of 0.75 mL/min.

3.6—

Synthesis of 6-Hydroxyhexyl β -[D]-Glucopyranoside Using Glucose as Donor (27)

1. α -[D]-Glucose (100 mg), 1,6-hexanediol (100 mg) and 30 μL citrate buffer pH 5.2, 50 mM, are mixed in a capped vial and heated up to ~95°C to dissolve the reactants.

2. The vials are then transferred to an incubator at 70°C and are kept at this temperature for 15–30 min.

3. The samples are then transferred to an incubator at 37°C. If a clear solution is not obtained, the vials must be reheated to 70°C for another 15–30 min. In some cases slight turbidity is still observed but, in our hands, this has no appreciable effect on the reaction rate or final yield.

4. The biotransformation is initiated by the addition of β -glucosidase (20 U in 20 μL 50 mM citrate buffer pH 5.2, containing 5 mM DTT).

5. The progress of the reaction is routinely monitored by gas chromatography (GC) or TLC by withdrawing samples (~2 mg) at specified time intervals. The samples are diluted with 100 μL of methanol and dried in a heating block (60°C) under a stream of nitrogen. If TLC analysis is used, the samples do not need drying.

3.7—***Synthesis of 6-Hydroxyhexyl β -[D]-Glucopyranoside and 8-Hydroxyoctyl β -[D]-Glucopyranoside Using Ethyl [D]-Glucoside as Donor (27).***

1. Ethyl glucoside (100 mg, anomeric mixture), 100 mg 1,6-hexanediol or 1, 8-octanediol and 30 μ L citrate buffer pH 5.2, 50 mM, are mixed in a capped vial and heated to $\sim 95^{\circ}\text{C}$ to dissolve the reactants.
2. The vials are then transferred to an incubator at 50°C and after incubation for 10 min the reaction is initiated by the addition of β -glucosidase (10 U in 20 μ L 50 mM citrate buffer pH 5.2, containing 5 mM DTT).
3. As above, the progress of the reaction is monitored by GC or TLC.

3.8—***Thin-Layer Chromatography***

1. Samples and appropriate standards are spotted on a TLC plate (silica gel 60) and the plate is developed with ethyl acetate:methanol (4:1 by vol).
2. After drying, the plates are sprayed with orcinol ferric chloride solution and dried for 5 min at 105°C . The spots appear blue to brown in color. This method is sufficiently sensitive to detect μg quantities of the glycosides.

3.9—***Gas Chromatography***

1. The dried samples are derivatized by adding 100 μ L BSTFA:pyridine (1:1 by vol) and 900 μ L dry pyridine in a capped GC vial, followed by incubation at 70°C for 30 min.
2. 1 μ L was then withdrawn for GC analysis, which is performed using a Hewlett-Packard GC series 5890 (Palo Alto, CA), fitted with a split injector (split ratio of 1:25), a fused silica capillary column (HP Ultra 2, Hewlett Packard, 25 m $\times$ 0.22 mm, 0.33 μm film thickness), and a flame ionization detector. The injector was held at 300°C , and the detector at 350°C . The temperature program is as follows: injection at 100°C ; ramp 20°C / min up to 325°C and held at 325°C for 15 min.

Acknowledgments

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26—

Use of β -Glucosidase in the Development of Flavor in Wines and Fruit Juices

Yannick Gueguen, Patrick Chemardin, Guilhem Janbon, Alain Arnaud, and Pierre Galzy

1—

Introduction

Flavor compounds synthesis by biotechnological processes today plays an increasing role in the food industry. This is the result of scientific advances in biological processes, making use of microorganisms or enzymes as an alternative to chemical synthesis, combined with recent developments in analytical techniques, such as HPLC, gas chromatography (GC), IR, or mass spectrometry. The study of the aromatic potential of some fruits, such as passion fruit, apple, and grapes as well as their fermentation products (juice, wine), has revealed that in addition to a free fraction of volatile terpenols, there exist naturally nonodorous and nonvolatile aroma precursors, which represent an important source of fragrant compounds (**1–3**). An important part of this aromatic pool is composed of terpenylglycosides, whose terpenic residue is β -glucosidically bound to disaccharide glucosides. The sugar moieties have been identified as β -D-glucose, 6-O- α -L-rhamnopyranosyl- β -D-glucopyranose, 6-O- α -L-arabinofuranosyl- β -D-glucopyranose, and 6-O- β -D-apiofuranosyl- β -D-glucopyranose. The aglycon part is frequently formed of terpenols, principally linalool, nerol, and geraniol with in some cases linalool oxides, terpenes diols, and triols (**4–6**). The presence of a β -glucosidic bond between the terpenic residue and the saccharide has suggested a possible liberation of terpenic molecules by enzymatic catalysis using a β -glucosidase. Such an enzyme has a great potential in the wine and fruit juices industry, since the quantities of bound monoterpenes in most fruit juices and wines are usually relatively high. Several studies were undertaken recently to develop the aromatic potential of monoterpene glycosides fully by enzymatic means (**7–9**). To be efficient in such a process, the enzyme should be active

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and stable at low pH value (pHs of wines and fruit juices are between 3.0 and 4.5) and active and stable in the presence of ethanol (wine). The enzyme should also be produced at high activity and, if possible, easy to isolate (extracellular). Moreover, a broad specificity for the enzyme is required to give the capacity to hydrolyze a great diversity of bonds between terpenols and aglycones, and consequently, increase the liberation of bound aromatic compounds. Janbon et al. (10) isolated a glucose-derepressed mutant able to produce large quantities of a β -glucosidase with interesting properties. The enzyme has a wide activity spectrum, an optimum pH of 3.5, and is very stable at low pH value (78% activity recovered after 145 h at pH 3.5, 30°C) (11). The enzyme is also stable in the presence of ethanol. Thus, the β -glucosidase from *Candida molischiana* 35M5N was chosen to develop an enzymatic system able to enhance the aromatic quality of wines.

2—

Materials

2.1—

Chemicals and Buffers

All chemicals should be analytical grade.

1. *p*-Nitrophenyl- β -D-glucopyranoside (Glc β Np) is purchased from the Sigma Chemical Co. The substrate solution is prepared in deionized water.

2. Citrate phosphate buffer: citric acid—dibasic sodium phosphate, 0.1 mol/L, pH 3.5.

3. Tartrate-phosphate buffer: tartaric acid—dibasic sodium phosphate, 0.1 mol/L, pH 4.5.

Sterilize buffers by autoclaving at 120°C for 20 min. Store at +4°C indefinitely. Ensure that cold solutions have equilibrated to room temperature before use.

2.2—

Culture Conditions.

The yeast strain *C. molischiana* 35M5N was isolated in our laboratory (10).

1. The basal culture medium is “G” medium (12). The different stock solutions used to prepare the this medium are the following:

a. Mineral salts: 10 g KH_2PO_4 , 60 g $\text{NH}_4\text{H}_2\text{PO}_4$, 20 g $(\text{NH}_4)_2\text{SO}_4$, 1 g NaCl, 5 g MgSO_4 , 7H₂O, 1 g CaCl_2 , 1000 mL H₂O. This solution is sterilized by autoclaving at 120°C for 20 min and stored at +4°C.

b. Vitamins: 80 mg calcium panthotenate, 80 mg thiamine, 80 mg inositol, 80 mg pyridoxine, 20 mg nicotinic acid, 0.8 mg biotin, 200 mL H₂O. This solution is sterilized by filtration with 0.45 μm Millipore sterile membrane and stored at +4°C.

c. Trace elements: 500 mg H_3BO_3 , 40 mg CuSO_4 , 100 mg KI, 400 mg MnSO_4 , 400 mg ZnSO_4 , 0.08 mg Na_2MoO_4 , 200 mL H₂O. This solution is sterilized by autoclaving at 120°C for 20 min and stored at +4°C.

d. Ferric chloride: 200 mg FeCl_3 , 1000 mL H₂O. This solution is sterilized by autoclaving at 120°C for 20 min and stored at +4°C.

Five hundred milliliters of basal “G” medium contain: 465 mL mineral salts; 5 mL trace elements; 25 mL vitamins; 5 mL ferric chloride. This basal medium is diluted 10 times in tartrate-phosphate buffer (0.1 M, 4.5) containing the carbon sources (cellobiose or glucose) at a final concentration of 0.5% (w/v) to make the growth medium (see **Note 2**). Glucose is used to start a liquid culture from a collection tube.

2. The cultures should be incubated at 28°C in Erlenmeyer flasks filled to one-tenth of their volume and shaken (80 oscillations/min, 8 cm amplitude).

3. Growth is monitored by measuring culture medium absorbance at 420 nm.

4. *C. molischiana* 35M5N cells are grown on cellobiose (0.5% w/v) until the end of the exponential phase (14 h). Then, the medium is centrifuged at 8000g for 20 min (at +4°C). The resulting supernatant contains the extracellular β -glucosidase.

2.3—

Enzyme Assay

β -Glucosidase activity is detected using *p*-nitrophenyl- β -D-glucopyranoside (pNPG) as substrate (**13**):

1. Add 0.1 mL of enzyme solution to 4.9 mL of 0.1 M, pH 3.5, citrate-phosphate buffer containing pNPG (5 mM final).

2. Incubate the reaction mixture at 30°C in a glass tube (10 mL). Take samples (0.5 mL) at regular intervals, and add to 1.0 mL of carbonate buffer (0.2 M; pH 10.2) in plastic cuvetts (see **Note 3**).

3. The liberated *p*-nitrophenol (pNP) in this mixture is assayed by spectrophotometry at 400 nm. The molar extinction coefficient used is 18,300 mol⁻¹/cm. One β -glucosidase activity unit (U) is defined as the quantity of enzyme required for hydrolysis of 1 μ of substrate (pNPG)/min (U/mL) under the above experimental conditions.

4. There are some small differences with the immobilized enzyme activity assay. To make the measurement easier and more accurate, the reaction volume used is 20 mL, and 0.1 g of resin is used/assay. The reaction was performed in a plastic tube of 30 mL (see **Note 3**).

2.4—

Enzyme Immobilization

The β -glucosidase of *C. molischiana* 35M5N is immobilized to Duolite A-568 resin (Rohm and Haas France S.A., Paris). Duolite A-568 is a highly porous granular weak base anion exchange resin based on crosslinked phenolformaldehyde polycondensate (principal functional group:tertiary amine).

The immobilization yields were calculated as following :

$$R = \frac{[(\text{Number of units put into contact with the carrier})/(\text{number of units retained by the carrier})] \times 100}{(1)}$$

2.5—

Gas Chromatography Analysis

GC analyses are performed using a Varian 3300 chromatograph (Varian Associates, Inc., Sunnyvale, CA) equipped with a flame-ionization detector

and a fused silica capillary column (15 m × 0.22 mm id, 0.25 µm film thickness) coated with DB1 (J & W Scientific, Folsom, CA) and an inlet system using the split (1:18) injection technique. Injector and detector temperatures are 200 and 250°C, respectively.

1. The column temperature is kept at 70°C for 1 min and then raised to 200°C at a rate of 1°C/min.
2. The carrier gas is helium at a flow rate of 2.7 mL/min.
3. The volatile compounds are primarily identified by comparing the retention times of the gas chromatographic peaks with those of commercial standards (Sigma). The identity of peaks is verified by GC-mass spectrometry using a Hewlett-Packard 5870 series II apparatus (Hewlett-Packard France, Les Ulis, France).
4. For each wine and fruit juice, the results represent the average of two experiments.

3—

Methods

3.1—

Enzyme Immobilization

The immobilization of a β-glucosidase provides a biocatalyst of potential value in releasing volatiles in fruit juice and wine. The advantage of using immobilized enzymes over traditional batchwise treatment for industrial process is primarily owing to a better control of the enzymatic process. Other advantages include the possibility of the repeated use of the biocatalyst and the feasibility of a continuous process.

1. Wash the Duolite A-568 resin with distilled water and then with 0.1 M citrate-phosphate buffer at pH 4.0.
2. Dry it overnight under vacuum.
3. Filter a culture supernatant (200 mL) containing 800 U of β-glucosidase activity (0.45 µm) to prevent contamination.
4. Without any concentration step, mix the filtered culture supernatant containing high level of β-glucosidase with the dry resin, and agitate for 1 h at room temperature.
5. Then, wash the resin sequentially with 100 mL of water (2X) and 100 mL (2X) 0.1 M citrate-phosphate buffer, pH 4.0. The immobilization is rapid (1 h), and the activity retained after immobilization is 82% of that of the original soluble enzyme.
6. Keep the immobilized enzyme at 4°C. In that condition, the immobilized β-glucosidase is stable over 1 yr.

The properties of the immobilized enzyme were studied and compared to the free enzyme (14) (see Note 4).

3.2—

Enzymatic Treatment and Flavor Extraction (2)

1. Add 50 U of immobilized β-glucosidase to 50 mL of wine or fruit juice obtained from the local market. Fresh fruits were used to make juices.

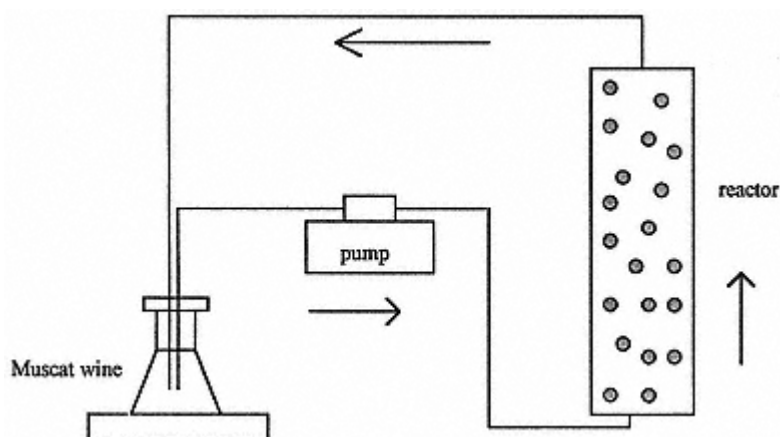


Fig. 1.

Scheme of the FBR for aroma precursors hydrolysis by immobilized β -glucosidase.

2. Run the enzymatic reactions in stoppered glass bottles (60 mL) at 30°C, with shaking using a magnetic stirrer.
3. After addition of 190 μ g of 4-nonanol (solution prepared in acetone) as standard, pass the wines and the fruit juices through a solvent-washed Amberlite XAD-2 column (1 cm id $\times$ 35 cm) with a flow rate of 2.0 mL/min (2). No pump is used.
4. Then, rinse the column with 100 mL of distilled water to eliminate sugars, acids, and other water-soluble compounds.
5. Elute the fractions containing free aroma by 50 mL of pentane/dichloromethane (2 v/1 v). The eluant is collected in a 100-mL Erlenmeyer containing anhydrous calcium sulfate to eliminate completely the water phase (see Note 5).
6. Transfer the eluate carefully to a 50-mL rotavapor flask, and concentrate to 50 μ L under reduced pressure (rotavapor), and subject 1 μ L to GC analyses (see Note 6).

The efficiency of the immobilized β -glucosidase was tested for several fruit juices and wines (see Note 7).

3.3—

Continuous Enzymatic Reactor for Aroma Precursors Hydrolysis

A fluidized bed reactor (FBR) may be used for the study of the hydrolysis of aroma precursors of Muscat wine of Lunel (pH 3.8, 15% alcohol) (Fig. 1, Table 1). In this reactor, the flow of substrate keeps the immobilized enzyme particles in a fluidized state. This continuous reactor is used in a recycle mode where the product stream is mixed with the incoming substrate stream. The use of the FBR provides a high catalytic surface area. For efficient operation, the particles should be of nearly uniform size. Otherwise, a non-uniform biocatalytic concentration will be formed up the reactor.

Table 1
Comparison of the Physicochemical Properties of the Immobilized and the Free β -Glucosidase of *C. molischiana* 35M5N

Property	Free β -glucosidase	Immobilized β -glucosidase
Activity immobilization, %	1/2	82
Optimal pH	3.5	3.5
Optimal temperature, °C	55	55
Activation energy (E_a), kJ/mol	45.4	38.3
K_m (pNPG), mM	0.2	0.6
K_i (glucose), mM	7.3	5.3

1. For the continuous process, load 400 U of immobilized β -glucosidase resin into the fluidized bed column reactor consisting of glass tube (total length 40 cm, internal diameter 2.5 cm) (**Fig. 1**).
2. The Muscat wine is recycled through a peristaltic pump at the bottom of the column for different time periods.
3. Maintain the flow rate constant (140 mL/min) with a peristaltic pump.

After the enzymatic treatment (**Fig. 2**), GC-MS analysis indicates increases in flavor compounds such as nerol, geraniol, linalool, γ -terpinene, 2-phenylethanol, and benzyl alcohol (see **Note 9**).

4— Notes

1. Ensure that all reagents are maintained at the appropriate temperature. In particular, the water bath and the solution must be preincubated at the right temperature for proper results (especially when you study the temperature optimum or denaturation of the enzyme).
2. The substrate (0.5% w/v) is first dissolved in the tartrate-phosphate buffer and then autoclaved, and just before starting a culture, the basal “G” medium is added (1/10 dilution). The complete “G” medium is not very stable. Contamination occurs very fast. It is better to store every solution separately and mix them before use.
3. Concerning the β -glucosidase activity assay:
 - Reactions were run for 2–4 min and samples taken every 30 s or 1 min, depending on the enzyme concentration.
 - Before reading the absorbance, mix the cuvet to obtain a homogeneous color and prevent air bubble formation inside of the cuvet. Moreover, absorbance must be read as soon as possible after the reaction. The pNPG substrate is unstable when exposed to the light.
 - Concerning the immobilized β -glucosidase, it is very important to maintain all the resin particles in suspension by mixing continuously the reaction tube during the reaction.
4. The optimum pH of both the free and the immobilized enzyme on Duolite A-568 resin was 3.5. The activity of the immobilized enzyme as a function of tempera-

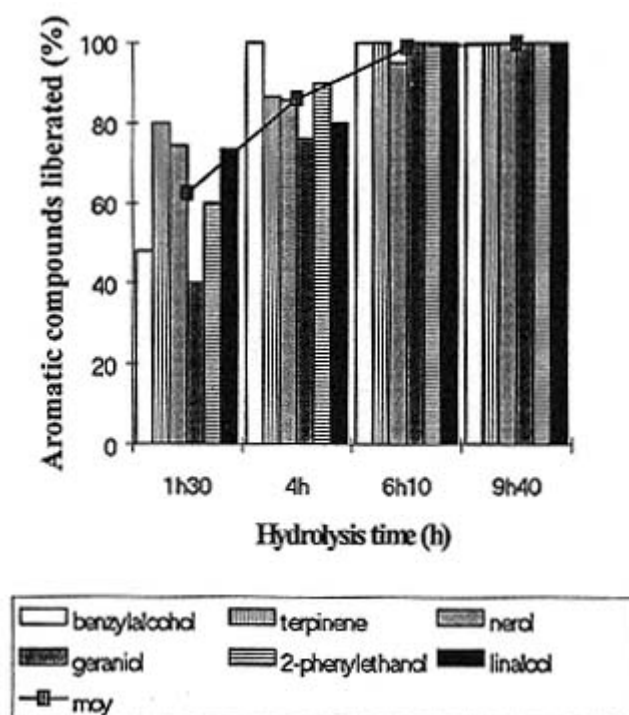


Fig. 2.

Effect of the use of the FBR on the aromatic compounds of Muscat wine flavor. Percent of the aromatic compounds liberated after different hydrolysis times.

ture was similar to the free enzyme (**Table 2**). The K_m value for pNPG for the immobilized enzyme was notably higher (0.6 M) than for the free enzyme (0.2 M). For β -glucosidase inhibition, two factors must be taken into account, i.e., ethanol and glucose. When pNPG was used as substrate, ethanol was an activator up to 1 M concentration, and 35% higher activity was obtained as compared to reference for both free and immobilized β -glucosidase. No inhibition was observed by 2 M ethanol in the medium (average value in wine). Glucose has been demonstrated to be competitive inhibitor for the free ($K_i = 5.3 \text{ mM}$) and the immobilized ($K_i = 7.3 \text{ mM}$) β -glucosidase.

5. It is possible also using this method to eluate the monoterpene glycoside (nonodorous aroma precursors) using ethyl acetate (2).
6. Gunata proposed in his protocol to filter the eluate (2). This step was shown to be not necessary if the separation between the eluant and the calcium sulfate is done carefully.
7. Determinations of the free volatile compounds (terpenes, cyclic alcohols) indicated that concentrations in enzyme treated fruit juices and wines increased significantly (14). For example, the free volatile compounds increased by 1250, 705, 141, and 12% in mango, strawberry, apple juice, and sauvignon wine. The results

Table 2
Experimental FBR Parameters for Muscat Wine Treatment

Parameters	Size
Weight of support, g	45
Reactor volume, mL	200
Total immobilized enzyme, U	400
Wine volume, l	1
Recycle flow rate, mL/min	140
Temperature	Room temperature

obtained are better than or identical to those obtained by the different researchers on the hydrolysis of aroma precursors (6–9). In this way, the use of β -glucosidases during winemaking or fruit juice processing can contribute to increasing greatly the amount of flavor compounds in wine and fruit juices and also improve their flavor quality.

8. After 6 MO of storage at +4°C, the activity of the immobilized β -glucosidase was 100% of the original activity. In addition, the stability of the immobilized β -glucosidase was investigated by measuring the residual activity after each enzymatic treatment in the fruit juices or the wines. One hundred percent of activity was recovered in all cases.

9. The immobilized β -glucosidase was found to be very stable during the treatment. It was found that there is no enzyme desorption during the process and that 100% of activity was retained at the end of the treatment. Sensory evaluations of the treated wine and fruit juice compared to the control were conducted. Nine of 10 judges identified correctly the treated Muscat wine and found a significant increase in the flavor and a richer Muscat flavor. In many cases, treated juice or wine was preferred, in particular, because of an intense fruitiness flavor.

10. To store the immobilized enzyme after a reaction in a fruit juice and reuse it later, take care that you have rinsed the resin several times with water (two times) and citrate-phosphate buffer 0.1 M (two times). If the washing step is not done with care, contamination can occur very rapidly. Some protocols proposed preserving the resin between uses or for long-term storage to dry it under vacuum. Use of this method resulted in the immobilized β -glucosidase of *C. molischiana* 35M5N losing most of its activity (70%) upon complete drying.

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